REMARKS

Support for the amendment to claim 37 can be found at page 13, lines 23-24. Applicants note that the Examiner has entered their amendments filed on 9 June 2000. Applicants further acknowledge the Examiner's return of a signed copy of the Information Disclosure Statement which they filed on February 22, 1999, with examined references initialed by the Examiner. Applicants still further acknowledge that the Examiner has removed the objections under 37 C.F.R. 1.821(d); and has further not repeated his rejections of claims 14-22 and 37-40 under 35 U.S.C. § 112, first paragraph (enablement and written description) and his rejection of claims 14-22 under 35 U.S.C. § 112, second paragraph (indefiniteness). The Examiner's remaining rejections are discussed below.

Claim Rejections under 35 U.S.C. §101

Claims 14-22 and 37-40 have been rejected under 35 U.S.C. §101 and 35 U.S.C. § 112, first paragraph because, the Office Action states, the claimed invention is not supported by either a specific and substantial or a well established utility. In particular, the Examiner states that: (a) "CAK1 is an orphan kinase with no known substrate activity or function;" and (b) "there is no nexus between the unknown properties of the CAK1 polypeptide and the diagnosis and treatment of systemic fungemia." In particular, the Examiner states that "further experimentation of the protein itself would be required before it could be used." Applicant respectfully traverse this rejection for the reasons which follow.

The application as filed provides guidance as to the credible, specific and substantial utility of the instant invention. First, the application points out that the nucleic acids of the invention can be used in diagnostic assays for detecting the presence of a *Candida* pathogen in a patient sample by means of a PCR-based procedure (see, for example the specification at page 9, lines 3-10 and at page 25, lines 17-29). This use of the *Candida* sequences of the invention is a credible and specific use of the invention because the isolation of a gene involved in an essential and evolutionarily conserved mitotic regulatory network provides a nucleic acid target which, distinct from the general class of cDNAs that might be isolated from *Candida*, is subject to stringent sequence conservation by virtue of its integral role in growth and development of this organism. Accordingly, this nucleic acid would provide a probe useful in detecting virtually any species of *Candida*. In contrast, a random cDNA from *Candida*, supplying no known function or a function unlikely to be subject to strict evolutionary conservation, would be unlikely to provide a useful specific probe for detecting *Candida*. Furthermore, this specific use of the probe of the invention is a substantial "real world" use of the instant invention because the need for accurate

and rapid diagnostic assays for detecting *Candida* infections is well-recognized and has received considerable attention (see e.g Lew (1989) Annu Rev Med 40: 87-97; Exhibit A). Accurate diagnosis of *Candida* infections can be particularly problematic. For example, diagnosis of *Candida* urinary infections is made difficult by the lack of traditional medical diagnostic criteria such as pyuria or a predictive threshold colony count (see e.g. Gubbins et al. (1993) Pharmacotherapy 13: 110-27; Exhibit B). Still further, the use of nucleic acid-based techniques for detecting the presence of *Candida* nucleic acid in a patient sample has been recently recognized as a unique approach to the medical monitoring of the spread of fungal infections; and for its unique potential to detect "deep-seated" mycoses in a noninvasive patient specimen by virtue of its extraordinarily high sensitivity (see e.g. Gottfredsson et al. (1998) Pathology 30: 405-18; Exhibit C). Accordingly, the application as filed teaches a credible, specific and substantial use of the nucleic acids of the invention as a probe for medical diagnostic assays.

Furthermore, the application teaches credible, specific and substantial uses of the proteins encoded by *Candida* CAK nucleic acids of the invention. The application teaches that the CAK kinase binds to and activates cyclin dependent kinase (CDK) so as to promote progression of the cell cycle and hence mitotic growth of the fungus (see e.g. page 6, lines 6-22 and SEQ ID NO. 14). The application further teaches a *Candida* cyclin dependent kinase (CAK) (see e.g. page 5, lines 11-27 and SEQ ID NO. 9). The application still further teaches that the encoded proteins of the invention, such as the CAK kinase, can be used to prepare anti-fungal compositions by generating dominant-negative mutants and subfragments of CAK (see e.g. page 22, line 33 to page 23, line 33) useful in inhibiting the mitotic grow of *Candida* by interfering with the interaction of CAK with CDK. Furthermore the application teaches that still other inhibitors can be readily obtained using drug screening assays analogous to those illustratively described for compounds targeting TYP1/ CDK complexes (see page 25, line 30 to page 30, line 7). Accordingly, the application teaches credible and specific uses of the CAK-encoding nucleic acids of the invention as specific targets for drug screening as well as a direct source of anti-fungal therapeutics in the form of dominant negative forms of CAK. Finally, the application teaches that *Candida* is a genus of fungus responsible for the majority of opportunistic pathogens in humans and that existing methods of treatment of *Candida* have many shortcomings (see e.g. page 2, line 27 - page 3, line 2). Furthermore, it is well recognized in the field that there is a strong need for anti-fungal agents in the health care industry (see e.g Klepser et al. (1998) Ann Pharmacother 32: 1353-61; Exhibit D). More specifically such anti-fungals have been recognized as essential in combating post-surgical infection (see e.g Patterson (1999) J Chemother 11: 504-12); in managing low birth weight infants (see Witek-Janusek (1998) Dimens Crit Care Nurs 17: 243-55; Exhibit E); and in treating susceptible cancer patients (see

e.g. Martino & Giremenia (1997) Curr Opin Oncol 9: 314-20; Exhibit F). Therefore there is no question that these uses of the CAK-encoded proteins of the invention are substantial, practical uses. Accordingly, the application teaches credible, specific and substantial uses of the CAK-encoding nucleic acids for generating CAK polypeptides useful in therapeutic applications as well as for direct use as nucleic acids in diagnostic applications as described above.

Still further, the CAK-encoded proteins of the invention have established uses as therapeutic anti-fungals useful in treating non-*Candida* fungal infections (see e.g. Whiteway et al. (1991) Proc Natl Acad Sci USA 89: 9410-14; Exhibit G). Whiteway et al. found that unaltered *Candida albicans* genes interfered with a *Saccharmomyces cerevisisae* signal transduction pathway involving the homologs of those *Candida* genes. Therefore the Whiteway et al. study demonstrates that the *Candida* CAK-encoding nucleic acids may be used to inhibit mitotic division in other fungi, such as *Histoplama capsulatum, Blastomyces dermatitidis, Coccidioides immitis,* and *Crytococcus neoformans.* Accordingly, in addition to the credible, specific and substantial uses of the invention, the CAK -encoding nucleic acids of the invention have well established uses as broad-spectrum antifungals that would be recognized by the skilled artisan in the field. Applicants therefore respectfully request reconsideration and withdrawal of the rejection based upon lack of utility under 35 U.S.C. § 101 as wells 35 U.S.C. § 112, 1st paragraph.

Claim Rejections under 35 U.S.C. §112, 2nd paragraph

The Office Action further states that claims 37-40 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention. In particular, the Examiner states that claim 37 "is indefinite for the recitation of stringent conditions, which is a relative term and it is not clear whether the condition is high, moderate or low stringency condition for hybridization." Accordingly, Applicants have amended claim 37 to recite specific conditions of hybridization of "2.0 x SSC at 50° C" as described on page 13 of the application as filed. Applicants believe this amendment obviates the rejection under 35 U.S.C. § 112, second paragraph and, accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

For the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of the pending rejections. Applicants believe that the claims are now in condition for allowance and early notification to this effect is earnestly solicited.

If there are any other fees due in connection with the filing of this Response, please charge the fees to our Deposit Account No. 06-1448. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit account.

Respectfully submitted,
FOLEY, HOAG, & ELIOT

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Exhibit A

DIAGNOSIS OF SYSTEMIC *CANDIDA* INFECTIONS

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ABSTRACT

Invasive candidiasis has become an important cause of morbidity an mortality in patients receiving aggressive antineoplastic chemotherapy, i recipients of bone marrow transplants, and in other patient groups. Th illness provides the clinician with few clues to its nature or presence. Th paper reviews developments over the past three decades in the laborato diagnosis of this important infection.

INTRODUCTION

The genus *Candida* was not defined until the 20th Century, but clinic syndromes attributable to it, notably oral thrush, have been describe since classical times (1). In the 19th Century, the designation "Monilia was applied to a variety of superficial infections associated with yeast-lik organisms, such as oral thrush and vaginitis. Although Robin recognize in 1853 that the thrush fungus could produce invasive infection as terminal event in other conditions, invasive candidiasis, defined as infectio of visceral organs by hematogenous spread, remained a medical curiosit until the mid-20th Century (1).

The principal factor governing the rise of invasive *Candida* infection since the 1950s appears to have been the widespread application of cyto toxic chemotherapy to the management malignancies. Bodey described startling increase in the incidence of invasive candidiasis between 1954 an 1964 at the National Institutes of Health, and identical trends were note

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at other oncology centers (2, 3). In the 1980s, invasive *Candida* infections are a major barrier to the success of aggressive antineoplastic chemotherapy and of bone marrow transplantation (4–8). In one recent series from a large oncology center, 11% of patients receiving treatment for acute leukemia developed invasive candidiasis, and evidence for this infection was demonstrable in 9% of autopsies (6). Advances in medical technology and improved control of bacterial infections have placed other populations at risk. These include surgical patients with complicated postoperative courses (9), patients receiving parenteral nutrition (10), and patients with extensive burns (11).

Clinicians caring for patients at risk for invasive candidiasis are painfully aware that there are few lethal infections that offer such a paucity of clues to their nature or presence. There is no characteristic syndrome associated with the illness, and because *C. albicans* can be recovered so frequently from the mucous membranes of healthy subjects, its presence in cultures from these areas offers little diagnostic help (12). The result is that, even in relatively recent series, as many as 70% of patients fail to receive timely therapy (6). Since the traditional tools of the clinician have been so wanting, much effort has been devoted to developing new diagnostic tests.

THE HISTORY AND PHYSICAL EXAMINATION

The clinician must maintain a high index of suspicion for invasive candidiasis for any high-risk patient who has fever that is unexplained or refractory to treatment with antibacterials (13). *Endophthalmitis* provides the only physical findings that are pathognomonic of systemic infection (14). Eye involvement occurs in 1–10% of patients with invasive candidiasis, but it may occur as a sequel to an otherwise harmless episode of "benign" fungemia (6, 14). Patients may complain of ocular pain, visual blurring, or scotoma, but a sizable minority have no symptoms. The most characteristic finding is one or more focal, white, mound-like retinal lesions, which may be associated with haziness in the overlying vitreous (14). If neglected, the infection may spread quickly to involve the entire contents of the eye.

Rash is another physical finding that may lead to timely diagnosis, but it, too, occurs in only a small minority of patients (6, 15, 16). Usually, the rash is nonspecific in appearance, consisting of small macules or slightly raised papules, sometimes with blanched centers (6, 14). It may be difficult to distinguish from other rashes, such as those caused by drug eruption, graft-versus-host disease, leukemic infiltrate, or infection by other fungi (6, 16). Unexplained skin lesions in the persistently febrile, neutropenic patient should be biopsied, since *Candida* lesions usually show invasion of

dermal blood vessels by yeast- and hyphal-phase organisms. These best demonstrated in sections stained by the PAS or silver method (16)

MICROBIOLOGY

The role of surveillance cultures of peripheral sites for early diagnosis invasive candidiasis was evaluated in a prospective study that monito 89 patients hospitalized for treatment of leukemia (17). Cultures of uri stool, and respiratory secretions were obtained twice weekly and pla on selective media for isolation of fungi. Recovery of *C. albicans* fr these sites had only 13% positive predictive value for concurrent invas infection. Surveillance for *C. tropicalis* was much more useful; isolati of this yeast from urine had a 73% positive predictive value for invasi infection, and recovery from urine or stool plus one additional site rais the positive predictive value to 83%. These data suggested that the att rate for invasive infection in patients who were colonized by *C. tropica* was inordinately high and implied that this species may be more virul than *C. albicans* for neutropenic patients. Failure to grow either spec in twice-weekly surveillance cultures had a negative predictive value >90% for invasive *C. albicans* or *C. tropicalis* infection. Colonization other yeasts occurred infrequently, often was transient, and did not reliab predict invasive infection. Clinicians who seek reassurance from negati surveillance cultures should be certain that the intensity and frequency their surveillance efforts and their culture methods closely resemble tho employed by these investigators (17).

Recovery of *Candida* from blood cultures is both insensitive and no specific for diagnosing invasive candidiasis. It has long been appreciat that candidemia, especially if it arises from infection of an intravenc catheter, can be a benign and self-limited event in immunologically norm hosts (18). Many such circumstances can be managed by withdrawal the offending catheter and careful observation for evidence of endop thalmitis or metastatic infection (18), albeit at some risk for late co plications, such as endophthalmitis, osteomyelitis, and endocarditis 19). In myelosuppressed patients, however, candidemia indicates a 90 likelihood of invasive infection, and prompt initiation of antifungal chem therapy is advisable in most such cases (18). The sensitivity of blo cultures is low, providing an antemortem diagnosis in only 14 to 33% patients, and the slow growth of *Candida* in conventional blood cultu media ensures that many positive cultures are recognized too late in t clinical course to influence patient management (4, 6).

Microbiologists have attempted to increase the yield and speed of fung blood cultures by catering to the aerophilic nature of these organism

Use of pour plates, venting of aerobic blood culture flasks, early blind subculture onto plated media, and vented biphasic blood culture flasks (containing a layer of agar and broth medium) all may produce modest improvements in recovery speed and sensitivity (20). Radiometric (20) and lysis-centrifugation (21) techniques appear superior to earlier methods, but the low levels of fungemia observed in experimental animals with invasive candidiasis suggest that the sensitivity of blood cultures will remain inadequate (22).

Diagnosis of candidemia by demonstration of organisms in Wright-Giemsa-stained smears of peripheral and central venous catheter blood has been described (23). Patients with this finding have had catheter-related fungemia or endocarditis, and it seems unlikely that this technique would have a significant yield in immunosuppressed patients, in whom the level of fungemia is assumed to be low. Recently, examination of urine sediment for fungal casts has been proposed for early detection of renal infection (24). Since the kidney is a major target organ in invasive candidiasis (4, 22), the diagnostic yield of this method should be assessed in high-risk patients.

SEROLOGY

The shortcomings of conventional microbiology have inspired numerous attempts to develop diagnostic tests that are based on the measurement of *Candida* antibodies. A large and generally disappointing literature on this subject has accrued over the past three decades. The interested reader is referred to the comprehensive review by de Repentigny & Reiss (25) for many more details than can be provided in this summary.

Mannan

Mannan is a major structural component of the cell walls of yeasts and the principal surface antigen that is available for immune interaction with colonized or infected hosts. It is a large-molecular-weight protein-polysaccharide whose carbohydrate portion contains backbone chains of repeating mannose units in α(1,6) linkage and numerous α(1,2)- and α(1,3)-oligomannoside side chains (26). Variations in the lengths of side chains, location of 1,3 bonds, and phosphodiester substitutions appear to determine antigenic specificity (25, 26). Whole-cell agglutination by specific antisera identifies two major serotypes of *C. albicans*, types A and B, and mannan comprises the type-specific antigen (27, 28). Serotype A accounted for 74% of isolates in one North American survey (29). *Candida tropicalis*, which is the second most frequent cause of invasive candidiasis, is serologically indistinguishable from *C. albicans* serotype A (28), and *Candida*

stellatoidea, a species encountered only occasionally in human infection is closely related to *C. albicans* serotype B (27).

Cytoplasmic Antigens

In theory, the presence of antibodies to cytoplasmic components should be more indicative of invasive infection than antibodies to mannan, since the former may be available to the host's immune system only during tissue invasion (30, 31). Since *C. albicans* converts from a yeast to mycelial phase during tissue invasion, cytoplasmic antigens that are unique to the latter should provide an additional measure of specificity (32). Investigators must make special efforts to remove mannan from their cytoplasmic preparations and demonstrate clearly that their reagents contain no carbohydrate and fail to cross-react with mannan antibodies (30–32). Secreted proteins or glycoproteins of cytoplasmic origin, such as exocellular enzymes, also provide potential targets for antibody detection (33).

A wide variety of assay techniques have been employed for measuring *Candida* antibodies. These include, in approximate order of sensitivity, agar-gel diffusion, whole-cell agglutination, passive hemagglutination, latex agglutination (LA), counterimmunoelectrophoresis (CIE), indirect immunofluorescence, radioimmunoassay (RIA), and enzyme immunoassay (EIA) (25). It is difficult to make interlaboratory comparisons of assay performance because of wide disparities in assay methods, patient populations, times of sampling, and, most critically, case definition. Proven cases of invasive candidiasis are difficult to obtain, since proof requires biopsy or autopsy demonstration of tissue invasion. Many studies stratify their populations according to level of diagnostic certainty, determined by clinical and conventional laboratory criteria, and leave the reader to place his or her own interpretation on the results.

Collaborative studies have shown that agar-gel diffusion, CIE, and LA provide approximately 80% sensitivity and specificity for diagnosing invasive candidiasis in patient populations with relatively intact immunity (25, 34, 35). Unfortunately, these tests lack sensitivity and the ability to distinguish colonization and superficial infection from invasive infection in immunosuppressed patients, whose capacity to mount humoral immune responses is limited (36). Application of very sensitive assay techniques, such as EIA, improves detection of antibodies but does not boost specificity to acceptable levels (37). Measurement of antibodies to cytoplasmic and mycelial-phase antigens also has failed to improve specificity (38).

In summary, antibody tests remain seriously flawed because the majority of healthy patients have antibodies to *Candida* mannan that can be detected in a variety of ways, but immunosuppressed patients, the population at greatest risk, respond poorly to antigenic challenge. This reduces the

sensitivity of antibody tests and makes it difficult to establish threshold titers that distinguish normal, colonized, and infected subjects. Perhaps future use of highly purified cytoplasmic antigens with the most sensitive assay methods will provide diagnostically useful tests, but for the moment tests based on other principles must be sought.

DETECTION OF CIRCULATING FUNGAL PRODUCTS

Diagnosis of invasive candidiasis by detection of products unique to the fungus in blood and body fluids has been the subject of two recent comprehensive reviews (25, 39). Clinical evaluation of newly developed tests has been hampered by the same scarcity of material from proven cases and variations in case definition and study design that have encumbered evaluations of antibody tests.

Mannan

Since mannan is the most abundant antigen on the surface of yeast cells, it is logical to search for it in the circulation of infected patients. The first demonstration of *Candida* mannan in serum was achieved by two-dimensional crossed rocket immunoelectrophoresis in a patient with chronic mucocutaneous candidiasis (40). Ironically, subsequent experience indicates that patients with this condition rarely manifest antigenemia (author's unpublished observations). A variety of more sensitive methods for detecting mannan have been developed, including hemagglutination inhibition (41, 42), CIE (43), RIA (44), EIA (36, 45–49), and LA (50, 51).

Reasonable generalizations about mannan detection can be made from the accumulated experience provided by published reports: (*a*) Mannan appears to circulate bound to serum proteins, which inhibit detection by immunoassays. Even in immunosuppressed patients, the bulk of this protein seems to be immunoglobulin (39, 47). (*b*) The thermal stability of mannan permits recovery from serum by heat extraction methods. These techniques generally involve dilution in alkali or buffer followed by boiling or autoclaving (37, 44–49), or digestion of serum by proteolytic enzymes, followed by heat inactivation (50, 51). (*c*) When extraction methods are employed, mannan can be detected in the circulation of a sizable percentage of patients with invasive infection. Reported sensitivities range from 47 to 100% (25, 39, 41–51). (*d*) Specificity is >90%, with very few patients experiencing colonization or superficial infection showing antigenemia (25, 39). (*e*) In many infected patients, mannan circulates at concentrations of 1–10 ng/ml, which is at or near the limits of detection of even the most

sensitive assay techniques, such as RIA or EIA (47). (*f*) Antigenemia may occur only intermittently during lethal infection, requiring that multiple serum samples be taken during the period of risk (47, 48, 51). (*g*) The serotype of the infecting strain of *C. albicans* may affect the performance of the assay; infections caused by serotype B may be detected less reliably because of the lower reactivity of this mannan to serotype A reagents (52).

Cytoplasmic and Uncharacterized Antigens

Two laboratories have reported detection of protein antigens that are presumably of cytoplasmic origin. In one study a sandwich RIA detected a protein in the sera of 12 of 19 patients with invasive *C. albicans* infection at concentrations of 0.5 to 1.6 μg/ml (53). No antigen was detected in 7 control patients, which included, interestingly, three patients with invasive *C. tropicalis* infection. A heat-stable protein of cytoplasmic origin was detected in the sera of seven patients with proven and nine of thirteen patients with suspected invasive candidiasis and was absent from 53 control sera (54).

Gentry et al (55) reported a latex agglutination assay that uses latex spheres coated with antibody derived from immune rabbit serum. Thirty of 33 patients (91%) with invasive *Candida* infections had agglutinin titers of ≥ 1 : 8, whereas zero of 100 control sera were positive at this level. The assay did not employ a heat extraction step, and the antigens that were detected have not been characterized. This method is now the basis of a commercial kit, which has been evaluated by several laboratories (50, 55, 56, 57). The sensitivity of the assay, using a titer of ≥ 1 : 4 as the criterion for a positive test, has ranged widely from 28% (50) to 66% (56). Specificity has been reported at >90%, provided that patients with circulating rheumatoid factor, which can falsely agglutinate antibody-coated latex spheres, are excluded (57).

In summary, fungal antigens are present in the circulation of a sizable percentage of patients with invasive candidiasis, and their detection shows great promise for improving diagnosis. There remain a number of obstacles to the widespread application of these tests. With the possible exception of latex agglutination, the ultrasensitive assay techniques required to detect *Candida* mannan are not readily portable to clinical laboratories. Large studies employing a bank of sera obtained prospectively at frequent intervals from patients with rigorously documented invasive infection are needed to learn more about the kinetics of antigen appearance and disappearance during infection and to establish whether serial monitoring of high-risk patients will provide diagnostic information that is sufficiently timely to influence patient management (25). At present, no such bank of sera exists, and there seems to be little prospect for funding the required collaborative effort. The available commercial latex reagents appear to

be of limited usefulness, since they detect antigen(s) that have not been characterized, have variable clinical sensitivity, and must be controlled rigorously for rheumatoid factor.

Metabolic Products

Detection by gas-liquid chromatography of metabolites in serum that are unique to *Candida* is another principle that has been exploited for diagnosis of invasive infections. Mannose may be detected as the free sugar (58), or it may be measured after hydrolysis of serum, where it derives from free mannose, circulating mannan, and, possibly, circulating glycoproteins (59, 60). The assay method is laborious and complex and requires rigorous internal standardization and preparation of volatile derivatives prior to chromatography. In the limited number of human subjects studied, sensitivity has ranged from 50 to 100% (58–60).

Arabinitol is a pentose produced in vitro by a variety of *Candida* species (61). It has no defined role in human metabolism, but low levels can be detected in the sera of some normal individuals, and the compound accumulates in the sera of patients with renal insufficiency (62, 63). The initial report in 1979 of arabinitol's detection in the sera of patients with invasive candidiasis (64) has been confirmed by subsequent observers (49, 62, 63, 65, 66). Reported sensitivities range from 38% (65) to 82% (64). In one study of 25 patients, correction for renal insufficiency by calculation of the arabinitol/creatinine ratio yielded a sensitivity of 64% and a specificity of 96% (63). The assay methods share the complexity of those used for mannose.

One laboratory has compared the diagnostic yields of gas-liquid chromatography for arabinitol or mannose and EIA for mannan (49). The sensitivities and specificities of arabinitol, the arabinitol/creatinine ratio, mannose, and mannan were, respectively, 26 and 87%, 13 and 93%, 39 and 87%, and 65 and 100%. The technology required at present for assay of metabolites is laborious and cumbersome and is beyond the capabilities of most clinical laboratories. It appears that this approach may be useful only if simpler and more rapid assay methods can be developed.

SUMMARY

At present the laboratory tools available to clinicians for the diagnosis of invasive candidiasis are limited. Surveillance cultures of peripheral sites have little predictive value, with the possible exception of *C. tropicalis* infection in neutropenic patients. Blood cultures, even when they are tailored for optimal growth of fungi, are slow, insensitive, and nonspecific. Measurement of antibodies to *Candida* is not helpful in immunosuppressed patients, who comprise the very group that is most vulnerable to invasive infections. Detection of circulating fungal products, particularly mannan, provides the desired level of specificity, but the clinical sensitivity of current assays is disappointing, and the technology for performing them is not readily portable to clinical laboratories. Commercially available latex agglutination kits detect uncharacterized antigen(s) and lack sensitivity and specificity. Funding for the research that might lead to improved tests seems inadequate. The clinician, who often is left to his or her own resources, must maintain a high index of suspicion and must be willing to treat empirically in high-risk situations (13).

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Candidal Urinary Tract Infections: A Comprehensive Review of Their Diagnosis and Management

Paul O. Gubbins, Pharm.D., Stephen C. Piscitelli, Pharm.D., and Larry H. Danziger, Pharm.D.

Despite its prevalence, the significance of candiduria remains uncertain. The pathogenesis of candidal urinary tract infections has been relatively well characterized and many risk factors have been identified. The disorders lack consistent diagnostic criteria, however, such as the presence of pyuria or a colony count above which is predicative of presence, location, or severity of infection. Treatment is unclear due to lack of data defining the natural progression of the disease. Although often recommended, it may not always be possible to remove risk factors. Amphotericin B, fluconazole, 5-flucytosine, and other antifungal agents are important agents for managing candidal urinary tract infections.

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Fungi are increasingly significant causes o nosocomial infections.[1–3] Data from the Nationa Nosocomial Infection Surveillance (NNIS) program indicate the proportion of nosocomial pathogens isolated that were fungi increased over the last 1C years.[1–3] *Candida* sp, particularly *Candida albicans*, accounted for the largest increases.[1–3] According to institutions including the Centers fo Disease Control, *C. albicans* is an increasingly important blood-borne pathogen.[4–5] The NNIS alsc suggests a probable increase in candidal urinary tract infections (UTIs) over the last decade,[5] a fac that is supported by prospective surveillance data from a large teaching hospital.[7]

Despite these trends, our understanding o certain nosocomial fungal infections such as candidal UTIs is not as complete as that of thei bacterial counterparts. In general, informatior concerning the natural progression of the infections is lacking. Although many risk factors are known and the pathogenesis is fairly wel characterized, questions concerning thei diagnosis and management remain. For example what is the significance of candiduria? Does i represents colonization or infection? With respec to management, are candidal UTIs self-limiting? Are they best managed by removing risk factors or is antifungal therapy necessary? If antifunga treatment is required, which agent is best and hov long should it be given? Until these questions are answered, at least in part, candidal UTIs wil continue to pose difficulties for clinicians.

Mycology

Fungi are indigenous to the environment, and it has been estimated that roughly 300,000 species exist.[3] Approximately 20 are associated with disease in humans.[9] Biologically, fungi are classified as eukaryotes; that is, they possess a membrane-bound nucleus and subcellular organelles within a cytoplasmic membrane composed of glycoproteins, lipids, and sterols.[10] Fungi are classified as either yeasts or molds. Morphologically, most yeasts are oval or round whereas molds are distinguished by branching, tubular processes called hyphae.[11] Certain fungi can grow as either mold or yeast.

Candida Species

Clinically *Candida* are opportunistic pathogens that affect only persons with altered host defenses.[11, 12] They are the most common cause of serious fungal infections.[13] Approximately 150 species of Candida have been described, but only 12 are associated with clinically significant infections.[8, 14] Morphologically, they are considered yeasts and are approximately 2.5–6 μ in diameter. They stain gram-positive, and their smooth, pasty white colonies resemble those of staphylococci.[9, 14] Microscopically, their size, the presence of budding, and possibly pseudohyphae easily distinguish them from bacteria.

Candida sp are normal human commensals throughout the gastrointestinal tract and the female genital tract. They have been cultured from the oropharyngeal cavity in 30% of healthy volunteers.[15] Generally, they are not considered laboratory contaminants.[14] Approximately 1–8% of all urine cultures from hospitalized patients yield yeast or *Candida*,[16, 17] with *C. albicans* accounting for over 50% of these isolates. *Torulopsis glabrata*, the second most commonly isolated yeast from urine, has been found in 5–33% of cultures positive for yeast.[16, 18–21] The organism was formerly classified as *C. glabrata*. It is distinguished from the *Candida* genus by the lack of mycelial growth.[20] Other non-*albicans* species reportedly occur in 3–28% of urine cultures positive for yeast.[18]

Candida albicans

Unlike many members of the genus, *C. albicans* is dimorphic and may exist as a budding yeast (unicellular form) or mycelial or pseudohyphae (filamentous form). This allows it to be differentiated rapidly from other *Candida* sp by the germ tube test.[21] An inoculum of 10^5–10^6 cells/ml of the organism is suspended in pooled human sera at 37°C for 1.5–3 hours. After incubation, the formation of small parallel-sided projections from the cell surface (germ tubes) is characteristic of *C. albicans*. When done correctly, the specificity of this test approaches 100%. The organism also may be distinguished from other species on the basis of fermentation reactions. It does not ferment lactose, and produces acid or acid and gas in carbohydrate media.

Non-*albicans* Species

The non-*albicans* sp that have been implicated in causing urinary tract infections include *C. lusitaniae*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, and *C. guilliermondii*.[11, 22, 23] The frequency with which individual species cause candiduria is difficult to quantify because speciation of isolates in the urine may not be routinely performed by many hospital microbiology laboratories. These organisms are differentiated from *C. albicans* by a negative germ tube test. It may be difficult to differentiate *C. lusitaniae*, *C. tropicalis*, and *C. parapsilosis* unless the appropriate biochemical tests are done, such as a cellobiose fermentation or a rhamnose assimilation test.[23] *Candida lusitaniae* is also differentiated by its morphology on cornmeal agar.[23]

Variable susceptibility to antifungal agents among the non-*albicans* sp has been reported. Due to the lack of standardized susceptibility testing and lack of data on the relationship between in vitro and in vivo activity, however, the clinical significance of this is difficult to assess at this time. In several limited case reports antifungal therapy has been successful.

Pathogenesis

Background

Historically, there has been confusion regarding the pathogenicity of *C. albicans*. Due to variable taxonomy, it is difficult to pinpoint initial reports. Whereas *C. albicans* was implicated in cases of oral thrush as early as the mid-1700s, the fungal etiology of this infection was not firmly established until the mid-1800s.[14, 20, 24] Over the ensuing 20 years, *Candida's* ability to penetrate tissue and gain access to the bloodstream and other end organs was demonstrated.[20] The first documented *Candida* infection in the urinary tract appeared in 1910,[26, 27] and since then the pathogen has been identified as a cause of urethritis, cystitis, and primary or secondary renal cortical infections in numerous reports (especially since 1940).[14, 27]

Yet, many continued to consider *C. albicans* saprophytic. In the mid-1960s it was suggested that the continued dismissal of it as a "harmless" saprophyte might lead to tomorrow's virulent pathogen.[28] With advances in medical technology and the increasing prevalence of the immuno-compromised host, it seems this prediction has come true. *Candida albicans* is now among the 10 most commonly isolated nosocomial

pathogens.[3] During the last decade the percentage of nosocomial infections caused by *C. albicans* rose from 2% to 5%,[3] and a significant increase in the isolation of *C. albicans* from the urine was observed.[3]

Definitions

Candida infections of the genitourinary system include primary renal candidiasis, symptomatic candidal cystitis, candidal urethritis, and asymptomatic candiduria. Similar to bacterial UTIs, they may be classified as upper and lower tract processes. The former refer to renal candidiasis, an infection of the renal tissue that may be either primary (lacking systemic involvement or a hematogenous source) or secondary (arising from a hematogenous source). The primary infection, a rare occurrence, is less common than secondary renal candidiasis and may actually be due to a subclinical candidemia.[20]

Symptomatic candidal cystitis, candidal urethritis, and asymptomatic candiduria are considered lower tract processes. Symptomatic candidal cystitis is an infection of the bladder with involvement of the urothelial surface. It commonly causes nocturia, frequency, and dysuria.[20] Candidal urethritis is a rare infection of the urethral tissues. Candiduria refers to the growth of *Candida* sp on culture of urine sediments collected on two occasions, separated by at least 24 hours, using proper collection techniques.[29] Asymptomatic candiduria is the presence of *Candida* sp in the lower urinary tract in the absence of tissue involvement. Clinically, it is difficult to distinguish between these processes without invasive procedures.

Morphology

Debate exists as to whether the yeast or mycelial form of *Candida* is more pathogenic. Initially, it was thought penetration of *C. albicans* through renal tissues necessitated a transformation from yeast to the mycelial form.[30, 31] Extensive work in this area produced contradictory results. The ability of tissue fluids to interconvert the two morphologic forms may have contributed to the ambiguity.[32] However, recent clinical reports implicating the yeast as a cause of significant disease, and studies using mutants of *C. albicans*, indicate both forms can invade tissues and proliferate.[32-34]

Lower Tract Infections

Currently, lower tract infections such as candidal cystitis are thought to result from an ascending infection with the urethra as the site of entry.[16, 35] Rarely, they can arise secondary to hematogenous candidiasis.[36, 37]

Upper Tract Infections

Unlike lower tract infections, the hematogenous route is considered the primary pathway for upper tract disease, as demonstrated both in animal models and clinically.[30, 31, 38-43] Clinically, renal candidiasis arises secondary to hematogenous candidiasis, and occurs in approximately 90% of patients with hematogenous candidiasis.[20, 38, 39, 43] Of 45 published cases of disseminated fungal infection, 40 patients had evidence of renal involvement.[39] In early animal studies, immunosuppressed mice injected intravenously with *C. albicans* demonstrated renal pathology similar to human renal candidiasis.[31, 40]

Renal candidiasis rarely results from an ascending infection.[14, 16, 38] Contrary to overwhelming evidence of this, however, limited human data suggest the disorder could develop from an ascending infection.[44, 45] The facts that hematogenous candidiasis occurs equally in both sexes, yet the frequency of renal candidiasis is higher in females, supports this possibility.[20] Furthermore, renal candidiasis has been produced in animals by inoculating the bladder with *C. albicans*.[46] In addition, the urinary tract can be the source for a secondary fungemia.[39, 46-51] In a retrospective surveillance of hospital-acquired fungemia, positive urine cultures were obtained prior to the development of several blood cultures positive for fungi in 58% of patients.[49] In more recent surveys of fungemia, the urinary tract was the portal of entry in only about 8%.[50, 51]

Translocation

Of interest, the gastrointestinal tract has been implicated as a portal of entry for secondary candiduria.[47] To determine if pathogenic fungi could be absorbed from the gastrointestinal tract unchanged, an investigator ingested a suspension of 10^{12} colonies/ml *C. albicans*. His blood cultures were positive 3 and 6 hours after ingestion, and he developed candiduria in approximately 3 hours.[47] This demonstrated the organism's ability to cross the intestinal mucosa and reach the bladder by hematogenous spread.

Risk Factors

In nearly 50 years of clinical experience numerous risk factors associated with the development of candiduria or candidal UTIs have been identified (Table 1). As with systemic fungal infections, they are usually related to either a drug- or disease-induced (neoplasms, diabetes) alteration in the normal host defense mechanisms. A recent small retrospective study of patients with candiduria due to *C. albicans* found risk factors similar to those described in previously published reports[26, 27, 33, 35, 39, 44, 52-67]; that is, previous antibiotic

Table 1. Frequency of Common Risk Factors for Candidal UTIs

Risk Factor	Number (%) of Case Reports
Antimicrobial therapy	41 (76)
Females	31 (57)
Males	23 (43)
Urinary tract abnormality	22 (40)
Diabetes	15 (28)
Presence of Foley catheter	6 (11)
Cancer	3 (5.5)
Immunosuppressive therapy	3 (5.5)

From references 25, 26, 33, 35, 39, 44, 53–67.

therapy. Foley catheterization, history of diabetes, and immunocompromised state.[52]

Host Defenses

The most basic defense mechanisms are mechanical barriers such as intact integument and mucosal membranes. Breakdowns in these barriers allow *Candida* sp to gain access to the bloodstream.

Once in the bloodstream, the immune defenses against Candida are complex. Several studies demonstrated human blood lacks direct candidacidal activity.[68, 69] Subsequently, the candidacidal property of blood was attributed to phagocytosis of fungi by leukocytes,[69–72] primarily neutrophils, monocytes, and eosinophils.[70–72] The efficiency of phagocytosis is limited by hyphal length, however.[69, 72] An additional mechanism for the candidacidal activity of neutrophils and monocytes is the interaction between the lysosomal enzyme myeloperoxidase and hydrogen peroxide.[73, 74]

Cellular immunity (T lymphocytes) and, to a lesser extent, humoral immunity are important in the natural host defense to candidal infections,[75] as supported by the prevalence of oropharyngeal candidiasis in patients with acquired immunodeficiency syndrome (AIDS). Separate investigators have demonstrated that the serum complement system is involved in the phagocytosis of *C. albicans*.[69, 70] Plasma contains heat-labile factors that enhance the phagocytic activity of neutrophils. When these serum complement factors are inactivated, leukocyte phagocytosis of *C. albicans* is decreased.[69, 70] Unlike their role in protecting the host against hematogenous candidiasis, the importance of these defense mechanisms in defending against genitourinary infections remains unproved.

Gender

Similar to bacterial UTIs, the frequency of lower tract *Candida* infections is higher in females.[20] In 54 case reports of fungal UTIs from 1946–1988, 57% of patients were women.[26, 27, 33, 35, 39, 44, 53–67] Conflicting reports suggest this predisposition may not be as marked as that of bacterial UTIs. Two-thirds of the patients in a prospective study of urinary candidiasis were female, although the male:female ratio was approximately equal in a retrospective study of predisposing factors in hospital-associated candiduria.[76, 77] Most likely, women are more predisposed than males due to anatomic factors, and the vagina being a reservoir for the organisms.[38]

Antibiotics

Broad-spectrum antibiotic therapy is a well-recognized risk factor for candidiasis. In approximately 76% of the 54 case reports of fungal UTIs from 1946–1988, the drugs were administered prior to the development of candiduria or candidal UTIs. Nineteen (32%) of 60 children receiving more than 5 days of systemic antibiotics had a positive urine culture for *C. albicans*.[78] Such figures are striking compared with the results of a study of 800 children receiving no antibiotics, in whom investigators failed to isolate *Candida* from a single one.[79] A prospective study examining the natural history of candiduria observed that 19 (65%) of 29 adults received prior antibiotic therapy and 16 of them had taken two or more agents.[76] In a case control study comparing predisposing factors in 20 patients with candiduria and 20 controls matched for age, sex, service, and diagnosis and/or procedure, patients were significantly different from controls in mean duration of prior systemic antibiotics.[77]

Antibiotics increase the frequency of candiduria in both pediatric patients and healthy adults. In one study, the frequency in children rose from 4% to 14% in boys and from 9% to 39% in girls after 2 weeks of therapy.[80] In healthy adults studied in this trial, the frequency increased from 6% to 11% in men and 20% to 33% in women after 1 week of antibiotic therapy.[80] It is of note, however, that the patients remained asymptomatic. The actual number or types of antibiotics may not be as important as the duration of therapy in general.

Several mechanisms have been proposed to explain this strong association, including the drugs' ability directly to enhance the growth of *Candida*. Although several in vitro studies demonstrated that the presence of certain antibiotics stimulates the growth of the organism, conflicting data have been published.[81–84] Another theory holds that antibiotics eradicate bacteria that secrete an antifungal substance or compete with fungi for nutrients.[85] The experiment in which a large inoculum of *C. albicans* ingested by a healthy volunteer translocated through the intestinal wall provided a model of the consequence of candidal overgrowth

due to elimination of such bacteria.[47]

Urinary Catheterization

It was surprising that an indwelling urinary catheter was present in only 11% of the 54 case reports of fungal UTIs from 1946–1988; however, many of the reports did not state the presence or absence of a catheter. In a recent study of 29 hospitalized patients with greater than 10^4 organisms/ml on routine culture, 18 (62%) had urinary catheters at the start of the study.[76] This differed significantly from the 10% found among the general hospital population when the investigators randomly sampled their institution on three separate occasions. According to a retrospective case control study, of 98 patients with candiduria, 56% had been catheterized within 1 week of a positive culture. In addition, the duration of catheterization was significantly longer in patients with than in those without candiduria.[77] In patients with candiduria the median duration of catheterization prior to a positive urine culture was 12 days, compared with 5 days in the controls.[77] Another case control study actually used the presence of an indwelling catheter as a matching criterion, since all patients with candiduria were catheterized.[86]

The risk of candiduria may be related to differences in the ability of certain *Candida* sp to adhere to the catheter. Although such differences have been demonstrated with respect to adherence to inert substances,[87] the data are sparse. In a study to quantify adherence, *C. tropicalis* adhered to both polyvinyl chloride and Teflon more extensively than *C. albicans*.[87] These results may apply to Teflon-coated Foley catheters; however, more data on the adherence of other *Candida* sp to Teflon are necessary before their clinical significance can be assessed. Furthermore, information on adherence to silicon and latex, both of which are used to make Foley catheters, is necessary before the relative risk of candiduria based on material can be determined.

Steroids and Immunosuppressives

In vivo data on the association of steroid use and the risk of developing candiduria or candidal UTIs are limited. Immunosuppressive agents such as azathioprine have been associated with the development of candidal UTIs, particularly after organ transplantation.[88, 89]

Studies performed in mice showed that cortisone enhanced candidal infections of animals regardless of the inoculum size.[31] Despite this enhanced infection, when the mice were infected with 10^4–10^5 organisms, there was no difference in mortality between the steroid-treated and control animals. When the inoculum size was increased to 10^6 organisms, cortisone significantly altered the course of infection. Differences in mortality were observed as well, and were marked in animals treated with a large dose of cortisone (0.5–2.0 mg).[31] These results were more remarkable when the inoculum size was increased to 5×10^6. The timing of cortisone administration also was important. Mortality was reduced (90% vs 70%) when steroid administration was delayed until 24 hours after infection with 5×10^6 organisms. As the delay increased, the difference between treated and control animals diminished. Thus the enhancement of infection caused by steroids is reduced if host defenses are given time to become established.[31]

Corticosteroids, often in combination with an immunosuppressive such as azathioprine or cyclosporine, may predispose patients to fungal infections by a mechanism similar to the in vitro model. These agents suppress the host's normal immune mechanisms against *Candida* sp to a level at which they may be overcome by the organisms. It has been suggested that this lowering of the host resistance causes a "virulence enhancement" of the fungus.[90]

Diabetes Mellitus

A history of diabetes was present in 28% of the 54 case reports of fungal UTIs from 1946–1988. In a study of the efficacy of flucytosine in candidal UTIs, 9 of the 30 patients had a history of diabetes.[91] In another prospective study of urinary candidiasis, 14% of the patients were diabetics, which was significantly higher than the 1.7% in all of the hospitalized patients dying or discharged over the course of 1 year.[76] Although the mechanisms by which diabetes may contribute to the fungal infections are unclear, it has been suggested that the increased amount of urinary glucose and increased residual urine associated with the inability to empty the bladder completely represents favorable conditions for the growth of *C. albicans*.[38] An in vitro study demonstrated that the growth and growth rate of yeasts in urine increase at urine glucose concentrations greater than 150 mg/ml.[92]

Surgery

The exact mechanism by which surgery is a risk factor for urinary candidiasis is unclear. Of 40 patients with persistent candiduria, 13 had undergone various types of procedures including vascular, malignancy, and bowel surgery.[93] In another series of 225 patients, 42 had undergone surgery.[94] These patients are most likely susceptible to candidal infection due to their underlying disease, presence of urinary catheter, or antibiotic use.

It is doubtful that any of these is the primary risk factor. Rather, all contribute to the alteration in the normal flora and host mechanisms that predispose to fungal overgrowth. Because the variables associated with candiduria and candidal UTIs are well defined, patients at risk can be readily identified. Unfortunately the diagnosis of this infection is less clear.

Diagnosis

The diagnosis of candidal UTIs cannot be made based on any specific laboratory tests. Unlike their bacterial counterparts, these infections lack consistent diagnostic criteria such as a critical colony count that identifies the presence, location, or severity of infection. Despite numerous case reports of urinary tract candidiasis, in the mid-1960s much controversy existed concerning the significance of positive urine cultures for *C. albicans*.[95] Since then researchers have attempted to define the significance of candiduria quantitatively.

In original studies, isolation of yeast from the urine of healthy volunteers or patients at low risk was extremely rare. These studies often compared populations at low risk with those at high risk to determine if there was a difference. Specimen-collection techniques were not always reported, but the results were similar. *Candida* or *Torulopsis* sp 10^3 cfu/ml or greater were cultured from clean-voided urine specimens of patients only 1% of the time (15/1500).[96] Only one patient had no reported risks. In a case report, "yeasts of any type" were isolated in only 1.7% (2/120) of clean-catch specimens. Both patients were receiving long-term antimicrobial therapy. The same report cited a series in which there was a high frequency of candiduria among patients at risk, but not a single isolate cultured from the urine of 800 healthy controls.[79] Low isolation of *Candida* sp from the urine of low-risk patients was also reported.[26]

More recently, in a prospective surveillance of clean-catch midstream specimens from 440 healthy adults, *C. albicans* was isolated in 10 (2.3%). On reculture, using proper collection techniques in 9 of the 10 subjects who returned for follow-up, no fungi were isolated.[97] The same study reported a very low frequency of candiduria among 317 patients with uncomplicated urinary tract pathology prior to surgery.[97] After surgery, however, the frequency rose. Others failed to isolate *Candida* sp from healthy individuals, yet among patients they were detected 6.5% of the time.[98] Given these data, it was believed that, regardless of the colony count, any fungal growth in a properly collected urine specimen was abnormal and indicative of an underlying pathologic progress such as systemic candidiasis.[96–98]

These results were disputed by researchers who believed that, similar to bacterial UTIs, there was a cutoff in colony counts that could differentiate between colonization and infection. In three separate reports it was demonstrated that even when using the most accurate methods of collection, *Candida* could be cultured from the urine in the absence of disease.[17, 80, 99] These studies determined that colony counts were variable and depended on the method of collection, and that to evaluate their significance adequately, the method of collection must be specified.

The first study reported findings from 30 patients who were actually part of a larger study. The authors reviewed records of patients with candiduria and autopsy reports documenting the presence or absence of histologically proved renal candidiasis.[17] Twenty patients had renal candidiasis and 10 did not. In 9 patients with renal candidiasis, specimens were from either a midstream clean-catch or a single urethral catheterization, and in the remaining 11 they were from a Foley catheter. The maximum colony count from midstream clean-catch or a single urethral catheterization was 40,000/ml, compared with 100,000/ml from a Foley catheter. In half the patients without renal candidiasis, specimens were from either a midstream clean-catch or single urethral catheterization and the remaining half were from a Foley catheter. The maximum colony counts were 15,000/ml and 100,000/ml, respectively. Variability was significant, particularly in specimens obtained from a Foley catheter. This suggested colony counts from midstream clean-catch or single urethral catheterization specimens were more reflective of the presence or absence of disease than those from Foley catheter specimens. It also suggested that colony counts greater than 15,000 from midstream clean-catch or single urethral specimens indicated infection.[17] This study lacked statistical analysis and it is unclear exactly how this cutoff was derived. It also demonstrated that in the presence of a Foley catheter, high colony counts suggest but do not indicate infection.

The second paper reported results of a larger study and included data from the original report. In this two-part study of colony counts urine specimens were obtained from a single urethral catheterization, a clean-catch specimen, or an indwelling Foley catheter.[99] Sixty-four patients with clinical or laboratory findings suggestive of renal candidiasis were retrospectively studied to determine the correlation between colony counts and the presence or absence of renal candidiasis. In these patients there were 20 histologically proved cases of renal candidiasis and 44 unproved. Candiduria was present in all of the histologically proved cases and in only 10 (22%) of patients without proved renal pathology.

This second report provided more complete data and statistical analysis than the first; however, some confusion exists concerning the colony counts. Colony counts were performed in 30 patients, 10 with and 20 without proved renal candidiasis, yet results for 34 patients (9 with and 25 without proved renal candidiasis) were reported.[99] In the original study,[17] samples obtained by a single urethral catheterization or a midstream clean-catch specimen were combined into the same group. To determine differences between the two collection methods, in the second report they were separated. The authors were able to detect a statistically significant difference in the mean urinary colony counts of catheterized specimens from patients with and without renal candidiasis. However, there was no difference in mean urinary colony counts when the specimens were collected by clean catch.[99]

The authors defined the cutoff between colonization and infection as the lowest colony count from a single urethral catheterized specimen in patients with proved candidiasis.[99] From these results the cutoff of 15,000, which had been used in the original report, was slightly modified to 10,000–15,000 colonies/ml. Furthermore, because counts from midstream clean-catch specimens were similar in the presence or absence of disease, it was recommended that greater than 10,000 colonies/ml from midstream clean-catch specimens be confirmed with a catheterized specimen.[99]

As in the first report, the presence of a Foley catheter promoted candiduria regardless of the presence or absence of disease. This suggested that colony counts reflected colonization of the catheter.[99] However, colony counts greater than $10^{2.5}$ obtained from patients with indwelling Foley catheters may represent the amount of *Candida* in the bladder and not merely colonization of the catheter.[100] In the second part of the study, 165 randomly selected inpatients were prospectively studied to determine the numerical relationship between colony counts in midstream and catheterized specimens. Colony counts in the latter were 2 to 5 times lower than in the former.[99]

A prospective study of 1004 urine samples attempted to determine the frequency of candiduria and the colony counts in asymptomatic patients and volunteers without signs of urinary candidiasis.[80] It showed that *Candida* could be isolated from the urine of asymptomatic and healthy children and adults. Due to study design, the specimens were not all collected in the same manner. They were all midstream specimens, but were not all obtained using a clean-catch technique. One hundred forty-nine midstream clean-catch specimens from 83 individuals without renal candidiasis obtained in this study were compared with similar specimens from six patients with histologically proved renal candidiasis from their previous work to determine if a cutoff between colonization and infection could be established.[99] Mean colony counts in patients without renal candidiasis were significantly lower than in patients with the disorder.[80] Coupled with previous findings, the authors concluded that colony counts of 10,000–15,000 from a midstream clean-catch specimen were rare, but that from a catheterized specimen they would serve as a useful cutoff between contamination and infection. In addition they reiterated that colony counts from catheterized specimens were more reliable than those from midstream clean-catch specimens.[80]

The absence of pyuria is also not helpful in ruling out the possibility of infection. In the retrospective study of patients with suspected renal candidiasis, pyuria was poorly associated with the presence of disease.[99] It was demonstrated in 61% of patients with histologically proved renal candidiasis, and in only 41% of those without proved histology. Because isolation of bacteria in the urine samples was not completely controlled for, the authors acknowledged that these data were limited. In a small study of *T. glabrata* urinary infections, pyuria was present in 8 (89%) of 9 patients with invasive disease, and only 25 (47%) who were believed to have asymptomatic colonization.[21] Eight of nine (89%) patients with invasive disease reported previous bacterial UTIs shortly before the study.[21] In patients with suspected asymptomatic colonization, bacterial cultures were incomplete. Although impressive, the results of this study are difficult to interpret without more information concerning the presence or absence of concomitant bacterial UTIs.

In summary, the isolation of *Candida* from urine should be considered abnormal and certainly warrants investigation. Although 10,000 colonies/ml may be a useful cutoff, it merely suggests the presence of renal pathology, it does not rule out its absence. There are many reports of patients with lower colony counts who developed serious candidal UTIs such as renal candidiasis, emphysematous prostatitis and cystitis, disseminated infection, and obstruction.[26, 33, 34, 57, 61, 67] Colony counts have never been predicative of the location or severity of infection. Although data are limited, they suggest the presence of pyuria also lacks predicative value. Unfortunately, the relationship between colony counts and the presence or absence of white blood cells in urine has not been adequately studied. Because of the nonspecificity of quantitative urinary fungal cultures, clinicians are often faced with a dilemma as to whether they represent colonization, infection, or an underlying hematologic candidiasis. Unfortunately, additional reliable, noninvasive laboratory studies for the diagnosis of candidal UTIs do not exist. In addition

to the amount of *Candida* in the urine, the presence of risk factors and prognosis of underlying disease must be considered when making decisions on how best to manage the patient.

Natural Progression of Candidal Cystitis

Tracking the progression of infection from the genitourinary system to the blood or vice versa is complicated.[97, 101] For example, it is difficult to isolate *Candida* from the blood, and it is possible that primary renal candidiasis is actually a result of a subclinical candidemia.[20] Unfortunately, few data exist to define the natural progression of this infection. Two studies addressed the natural progression of candidal cystitis.[17, 76] The first, a retrospective study, evaluated case histories of 82 patients with 1.5×10^4 cfu/ml or more of yeast.[17] Patients were classified into one of three groups. Group 1 (37% of the study population) included patients with no serologic evidence or systemic findings of infection, who received no antifungal therapy, but were treated with nonspecific measures such as controlling underlying diseases and risk factors. Most patients in group 2 (21%) had serologic evidence of infection, but none received antifungal therapy. Group 3 (42%) consisted of patients who had serologic evidence of infection and received antifungal treatment. Group 1 patients had no mortality, and candiduria resolved given time and control of underlying risks. Based on serologic findings it was noted that many group 2 patients should have received antifungal treatment. Nine (53%) of these patients died and eight (47%) improved with reduction of risk factors. All patients in group 3 had several positive culture sites and were initially treated with flucytosine. Five (14%) died despite therapy. The investigators concluded that, based on the differences in mortality between treated and untreated patients, if left untreated, candiduria will persist, disseminate, and contribute to mortality.[17]

The design and conclusions of this study are of concern. The definition of patients was very subjective, especially in group 2. The analysis of patients in group 2 was poorly reported. It is not clear why some of these patients, especially the eight who lived, were not included in group 1. It is also not stated whether the nine deaths due to complications in group 2 were related to candidal UTIs. The conclusion mentioned dissemination of infection, yet progression of infection from urine to the blood stream or vice versa was not reported, and it is unclear how this was determined. The authors also based their conclusion on differences in mortality among groups. However, the groups were not comparable demographically, nor were underlying diseases and the causes of death reported. Furthermore, the mortality rate of untreated patients (group 2) did not include the 30 patients who received nonspecific treatment (group 1). Had these patients been included in the untreated group, mortality would have been 19%, similar to that of the treated group (14%). The findings of this study differ from those of a case-controlled study demonstrating that although candiduria was associated with increased morbidity, it was not associated with increased mortality.[77]

In a recent prospective study, the resolution rate was 65% in the absence of therapy.[76] This was considerably higher than the previously reported figure of 37%.[17] No cases of hematogenous candidiasis were observed. Eight patients (28%) died, although in all cases, death was attributed to causes other than fungal infection. Autopsies were performed in two patients and revealed no evidence of renal infection. Forty-one percent of the isolates were non-*albicans* sp, and the high spontaneous resolution rate may reflect a lower degree of virulence associated with these organisms compared with *C. albicans*.

Treatment

Treatment of lower and upper UTIs due to *Candida* must be discussed separately. Because renal candidiasis often represents a process secondary to an underlying systemic infection, local therapy is not indicated. The treatment of lower tract infections is not as clear.

Nonpharmacologic Treatment

The first step in treating lower candidal UTIs is to remove any predisposing factors. Maneuvers such as removing or replacing indwelling urinary catheters and discontinuing unnecessary antibiotics and corticosteroids may be tried. Often, however, they may be contraindicated. Furthermore, *Candida* colony counts may continue to increase despite removing or replacing an indwelling urinary catheter.[100]

Pharmacologic Treatment

Prior to the development of amphotericin B (AmB), treatment of candidal UTIs was generally ineffective and consisted of administration of sulfa or penicillin antibiotics combined with bladder irrigations using a variety of alkaline solutions.[27, 53, 54, 102] In 1946, a case of candidal cystitis that persisted over 3 months was reported.[27] During this time the patient was treated with a variety of therapies, of which only urinary alkalinization was perceived to be beneficial. Two years later, urinary alkalinization was ineffective in a patient with marked *C. albicans* cystitis.[53] These cases suggest candidal cystitis may resolve without

Table 2. Case Reports of Intermittent Amphotericin B Irrigation in the Management f Candidal UTIs

No. of Pts.	Additional Antifungals	AmB Concentration	Irrigation Site	Duration
2	None	15 mg/100 ml SW	Bladder	11 days
	None	15 mg/400 ml SW	Bladder	8 days
2	AmB i.v., nystatin p.o. and topically	50 mg/100 ml	Renal pelvis	14 days
	AmB i.v.	100 mg/500 ml	Renal pelvis	Unspecified
1	None	25 mg/12.5 ml SW + D_5W	Urethra	Three treatments
6	None	100 mg/500 ml SW	Bladder	6–10 days
1	None	50 mg/L	Bladder	8 days
3	FC 20 mg/kg x 8 wks	30 mg/20 ml SW	Bladder	14 days
	None	15 mg/20 ml SW	Bladder	15 days
	None	15 mg/20 ml SW	Bladder	10 days
	FC 50mg/kg	Not used	NA	NA
	None	15 mg/20 ml SW	Bladder	20 days
	FC 100 mg/kg x 6 wks	Not used	NA	NA
	AmB i.v. 611 mg/17 days	20 mg/20 ml SW	Bladder	17 days
1	None	20 mg/200 ml D_5W	Renal pelvis	7 days

F/U = follow-up; FC = flucytosine: AmB = amphotericin B; NA = not applicable: SW = sterile water; (-) = urine culture negative for candidal growth; (+) = urine culture positive for candidal growth.

therapy over time.

Nystatin, an antifungal similar in structure to AmB, was recommended as an alternative for bladder washes,[102] and was associated with modest success.[62, 103, 104] However, it never gained wide acceptance due to poor water solubility and instability.

Amphotericin B

This is one of two antifungal extracts obtained from an anaerobic actinomycete, *Streptomyces nodosus*. It is a heptaene macrolide antibiotic that has notoriously poor aqueous solubility and is considerably toxic when administered intravenously. Other polyene macrolides have been isolated, but AmB is the only one currently used in clinical practice.

The agent acts by binding to sterols, particularly ergosterol, present in the plasma membrane of susceptible fungi. The resulting interaction increases the permeability of the fungal cell wall, which eventually leads to lysis. In mammalian cells the primary sterol is cholesterol, to which AmB binds with less affinity compared with ergosterol.[105] The drug is poorly absorbed after oral administration and undergoes minimal renal elimination.[105]

Because of its minimal urinary elimination and the toxicities associated with intravenous therapy, AmB has been employed as a urinary tract lavage for the treatment of candidal cystitis. It is either instilled intermittently or infused continuously into the genitourinary system. It is difficult to compare the two modes of administration. Data concerning the intermittent regimens are available only in case reports, and a randomized, prospective comparison of the methods has not been published. Intermittent regimens use concentrations and lengths of treatment that differ from those of the continuous regimen, and in half of the case reports describing intermittent administration, it is adjunctive to systemic therapy for complicated renal candidiasis.[26, 64, 106–109] For these reasons it is best to consider the two modes of administration separately.

Intermittent instillations. Amphotericin B was first used as a urinary irrigant by Goldman et al in 1900.[26] Since then several case reports have described the treatment in which the drug is instilled into the renal pelves, bladder, or urethra, generally once or twice/day (Table 2).[23, 26, 64, 106–109] Concentrations ranged from 0.037–2 mg/ml, with

Table 2. **Case Reports of Intermittent Amphotericin B Irrigati n in the Management of Candidal UTIs (continued)**

Time to Urine Sterilization	Comments
Day 5	Avg. retention time 1 hr; urine also alkalinized; F/U at 5 mo urine (-).[26]
Day 2	Avg retention time 20 min; F/U at 1 mo urine (-).[26]
Day 5	Total AmB i.v. dose 610 mg; F/U at 6 mo urine (-).[107]
Unspecified	*C. krusei*; pt. relapsed x 3, subsequent therapy failed to eradicate organism. Pt. died during final treatment; no F/U available.[107]
Day 7	Retention time 15 min; during second course pt. experienced pain and subsequent dose halved. F/U at 3 mo (-).[108]
Day 10	Two vesical washings/day using 100-ml solution. Pt. instructed to retain solution for 2–3 hrs. F/U urine (-) several weeks. 1 case persisted several mo. Pt. received only 3 washings.[109]
Day 8	20-ml solution instilled q3h. Pt. instructed to retain solution as long as possible. F/U urine (-) at 4 mo.[23]
Unspecified	F/U urine (-) after FC.[64]
Unspecified	F/U urine (+) at 3 mo. Second treatment given, F/U urine (-) at 3 mo and 4 yrs.[64]
Unspecified	
Unspecified	Symptoms not improved 6 wks.
Unspecified	Symptoms improved slightly.
Unspecified	Little improvement at 6 wks.
Day 17	Marked improvement; by end of treatment urine (-).[64]
Unspecified	Clear at 2 mo[24]

F U = follow-up: FC = flucytosine: AmB = amphotericin B: NA = not applicable: SW = sterile water: (-) = urine culture negative for candidal growth; (+) = urine culture positive for candidal growth.

little report of discomfort or adverse effects.[23, 26, 64, 106–109] Usually, 20–100 ml was instilled and retained by the patient for 1–3 hours as tolerated.[26, 64, 109] In other reports, larger volumes (400–500 ml) were instilled and retention times were much shorter.[26, 107] The duration of therapy varied, ranging from 5–20 days.[23, 26, 64, 106–109] Due to the complicated nature of some of the infections, several patients required repeat courses.[64, 107] When reported, the urine was culture negative at 2–10 days.[23, 26, 107, 109] Follow-up ranged from 1 month–4 years, and in general patients did not relapse or become reinfected.[23, 26, 64, 107–109]

From these case reports the treatment appears to be effective and well tolerated. It is suggested that AmB 15–30 mg in 100 ml sterile water be instilled into the bladder daily for 5–7 days. The patient should retain this volume for at least 1 hour or as tolerated. Patients who would not be candidates for this therapy include those who are unconscious or incontinent.

Continuous irrigations. Continuous bladder irrigations (CBI) of AmB have been effective in treating upper and lower tract infections.[57, 61, 62, 93, 95, 110–113] It has been used alone, and in combination with intravenous AmB, increased hydration, local and systemic alkalinization, or additional genitourinary surgery (Table 3).[57, 61, 62, 95, 111, 112]

Ten patients with indwelling urethral catheters and persistent candiduria received 5 days of AmB 50 mg/L CBI.[110] Half of them initially received 5 days of sterile water bladder irrigation prior to the 5-day course of AmB CBI. Candidal cystitis was eradicated in 70%. The three who failed had clinical and serologic evidence of disseminated infection and subsequently received systemic therapy. Serum levels of AmB were monitored and demonstrated minimal systemic absorption from the bladder.[110]

Several years later the same investigators reported a larger series in which 40 patients received AmB 50 mg/L CBI for 4–14 days.[93] In 37 (92.5%) candiduria cleared. Two of the failures had serologic evidence of dissemination and were subsequently treated with systemic AmB, which resolved the disorder. Serum levels in nine patients ranged from less than 0.04 μg/ml to 0.08 μg/ml, indicating that AmB absorption from the bladder mucosa was minimal. Follow-up cultures were obtained in 14 patients and documented relapse in 6 (43%). In the other 23 patients, verbal follow-up of the 19 survivors disclosed no overt urinary pathologies. It was concluded that AmB

Table 3. Case Reports and Clinical Trials of Continuous Amphotericin B Irrigation in the Management of Candidal UTIs

No. of Pts.	Additional Antifungals	AmB Concentration	Site of Irrigation
1	None	50 mg/L	Renal pelvis
1	AmB 141 mg i.v./SWI	50 mg/L	Renal pelvis
	None	50 mg/L	
	AmB 160 mg i.v.		
2	None	70 mg/L	Renal pelvis
	None	35 mg/L	Renal pelvis
	None	75 mg/L	Renal pelvis
	None	50 mg/L	Ureter
1	None	10 µg/kg/hr x 5 days; 2 µg/kg /hr x 5 days	Renal pelvis
1	Nystatin by nephrostomy tube	Unspecified	Ureters
2	FC	50 mg/500 ml SW	Bladder
	None		
10	SWI in 5 pts. x 5 days	50 mg/L SW	Bladder
40	FC added in 2 AmB CBI failures (both resolved)	50 mg/L SW	Bladder
65[a]	None	50 mg/L	Bladder

[a]This represents the number of cases in 54 patients.
F/U = follow-up; FC = flucytosine; AmB = amphotericin B; CBI = continuous bladder irrigation; SWI = sterile water irrigation; SW = sterile water; (-) = urine culture negative for candidal growth; (+) = urine culture positive for candidal growth.

CBI appeared to be a safe, effective treatment for noninvasive candiduria and could prevent the development of hematogenous candidiasis.[93]

More recently, the minimum duration of AmB 50 mg/L CBI was studied.[113] First, an in vitro *Candida* susceptibility test calculated the theoretical mucosal concentrations of AmB 50 mg/L CBI, accounting for dilution due to the patient's inherent urine production. At AmB concentrations of 25 and 5 mg/L, there was no growth of *Candida* after 48 hours of incubation. These figures represent the theoretical concentrations of drug in the bladder for a patient administered 50 mg/L AmB CBI with a 24-hour urine output of 1 and 9 L, respectively. From this in vitro work the authors derived a 2-day treatment regimen and studied it prospectively.

Fifty-four patients were treated with AmB 50 mg/L in sterile water or D_5W CBI for 48 hours, through a newly inserted indwelling urinary catheter. Immediately after the CBI, the bladder was washed with 5% dextrose in water to remove residual AmB, and fresh urine samples were obtained for culture and analysis. Sixty-five cases of confirmed candiduria in 54 patients were studied. In 47 cases (72.3%), *Candida* was eradicated, in the remaining 18 (27.7%) the yeast persisted. In 10 of the 18 failures, an additional course of irrigation for an average of 7 days was given, but in all 10 the yeast persisted. Of the 47 treatment successes, follow-up cultures were obtained in 31, 1–58 days after the end of therapy. In 24 patients the urine remained clear. It was recommended to shorten the duration of therapy from the standard 5 days to 2 days. The investigators pointed out that the additional therapy given to the patients who failed treatment demonstrated that failure was not due to inadequate treatment. Rather, it may have represented deep-seated infections that were not amenable to local therapy.

The 2-day regimen produced a low eradication rate (72%) coupled with the relatively high failure (27%) and relapse rates (22%) compared with data from the trial using longer treatment.[93] In the latter, therapy averaged 6 days, and there were only three failures (7.5%). Furthermore, although not well documented, only two (5%) patients relapsed. In addition, the inclusion of patients more than once in the study evaluating the short regimen creates confusion. It is unclear why patients who were studied twice were not considered relapses. It is also not clear if the 10 persistently positive cultures represent 10 patients or cultures. Finally, the outcome of the remaining eight persistent cases was not addressed. In light of this, routine use of a 2-day regimen cannot be recommended at this time.

The investigators suggested the 2-day regimen be used to differentiate upper tract infection from lower tract infection. It was thought that the immediate sampling of the post-irrigation urine specimen precluded reinfection or emergence of

Table 3. Case Reports and Clinical Trials of Continuous Amphotericin B Irrigation in the Management of Candidal UTIs (continued)

Duration	Day to Urine Sterilization	Comments
- days	Day 1	F/U at 1 mo, urine (-).[95]
9 days	Day 6	F/U at day 10 urine (+).[57]
15 days	Day 8	Urine alkalinized; F/U at day 20 urine (+).[57]
		Cultures (-); no F/U given.[57]
10 days		F/U at 6 mo; urine(-).[61]
10 days		*C. krusei* isolated; urine(+) day 11; dosage adjusted;
10 days	Day 20	F/U not addressed.[61]
10 days	Not addressed.[111]	
10 days	Day 2	Cultures (-) during 2-yr F/U.[112]
Unspecified	Unspecified	FC added after AmB CBI failed; F/U not addressed.[62]
2 days	Day 2	Patient underwent right pyelotomy prior to AmB CBI; urine cultures (-) during 1-yr F/U.[62]
10 days	Not addressed	7/10 patients responded; F/U not addressed.[110]
4–14 days, average 6 days	Therapy stopped when urine clear	Eradicate 37/40; persist 3; 1 resolved with catheter removal. F/U in 14, cultures negative at 4 wks in 8; significant bacteriuria in 4; colony counts < 5000 in 2.[93]
48 hours	2 days in 47/65	Eradicate 47/65; F/U in 31, day 3–60 days, 24/31 remained (-); persist 18/65; 10/18 persist with further CBI AmB.[113]

This represents the number of cases in 54 patients.
F/U = follow-up; FC = flucytosine; AmB = amphotericin B; CBI = continuous bladder irrigation; SWI = sterile water irrigation; SW = sterile water; (-) = urine culture negative for candidal growth; (+) = urine culture positive for candidal growth.

invading *Candida* from the submucosa of the bladder. They theorized the 18 treatment failures represented upper tract infection or colonization. There was no mention of whether or not any of these individuals had hematogenous candidiasis, and no invasive or radiologic studies were performed to confirm the possibility of upper tract disease. For these reasons, further investigations using invasive procedures are required before this method can be recommended to differentiate upper from lower tract infection.

In summary, local intermittent or continuous irrigation with AmB, alone or in combination with other systemic antifungal agents, appears to be safe and useful in the management of candidal UTIs. Due to the poor follow-up in the reported trials, its efficacy has to be defined, and questions concerning the associated relapse rate remain.

Intravenous Amphotericin B. Several case reports and two small clinical studies described intravenous AmB for the management of candidal UTIs.[62, 66, 114, 115] Preliminary results of a very small clinical trial produced encouraging data regarding a single low dose in four patients with persistent candiduria.[114] Before enrollment, all risk factors were minimized when possible, including changing the indwelling urinary catheter if present. All patients tolerated a 1-mg test dose, after which they were given a single dose of 0.3 mg/kg over approximately 4 hours, which also was well tolerated. Cultures became negative in 75% of patients and remained negative for a range of 10 days–11 months. The one failure had a long-term indwelling Foley catheter. Drug concentrations were detected in the urine for 5–14 days. It was theorized that the success of the low dose was due to the prolonged renal excretion of AmB.

Intravenous liposomal AmB (LAmB) was effective in treating complicated UTIs such as renal candidiasis due to *C. albicans*.[15] Patients received 50 mg/day (total 312–400 mg) LAmB over 7–10 days. Urine typically became culture negative in 1–4 days. Follow-up lasted 4–10 weeks. One patient relapsed at week 10 and received an additional 10-day course (500 mg total). This patient remained culture negative during 8-week follow-up. Side effects and renal toxicity were minimal. Patients received no premedication and tolerated rapid infusion over 15–30 minutes. Although LAmB was effective in complicated UTIs and associated with minimal toxicity, it is investigational and not currently available.

Systemic AmB may be an effective treatment of candidal UTIs in certain patients despite its low renal elimination. However, the products used in studies are markedly different. Because of the associated renal toxicity of the currently available AmB product, in the absence of renal candidiasis, regimens using several intravenous doses cannot be recommended.

Table 4. Clinical Studies of Flucytosine for Candidal Urinary Tract Infections

No. of Pts.	Route	Duration (days)	Dosage (mg/kg/day)	Clinical Response	Comments
225	Oral	21–28	100–200	94%	Secondary resistance developed in 6% of patients.[94]
30	Oral	28–42	100	77%	In most cases, cultures negative within 2–3 wks after start of therapy.[31]
37	Oral	7–14	8 g/day	77.8%	Secondary resistance in 6 patients with poor response.[123]

Flucytosine

Flucytosine (FC), a fluorinated pyrimidine related to fluorouracil, has been effective in the treatment of fungal infections due to *Candida*, *Torulopsis*, and *Cryptococcus* sp for nearly 30 years.[116] In fungi, FC is deaminated to 5-fluorouracil (5-FU) by the enzyme cytosine deaminase. 5-Fluorouracil is further metabolized to 5-fluorouridylic acid by the enzyme uridine monophosphate (UMP) pyrophosphorylase. 5-Fluorouridylic acid is then either incorporated into fungal RNA where it interferes with protein synthesis,[19] or further metabolized 5-fluorodeoxyuridylic acid, which impairs DNA synthesis.

After oral administration, FC is approximately 90% absorbed.[20] It is primarily excreted in the urine, and concomitant urine levels are approximately 10–100 times those in the serum.[117] To ensure adequate urinary levels throughout the dosing interval, patients should be monitored to achieve serum concentrations between 25 and 120 µg/ml.[20, 118] Due to its significant renal elimination, dosage adjustments are recommended in patients with renal impairment.[20, 118] The usual dosage in patients with normal renal function is 150 mg/kg/day in four divided doses, although many clinicians begin therapy for UTIs at 100 mg/kg/day. Although it is relatively well tolerated, side effects that may occur are nausea, vomiting, diarrhea, bone marrow toxicity, azotemia, and increases in liver enzymes.[118–120] Mammalian cells cannot convert FC to 5-FU; however, the microbial flora in the human intestine are capable of performing this conversion.[121] It is believed this mechanism may be responsible for the observed toxicity profile.[121, 122]

Flucytosine has been used successfully in the treatment of urinary candidiasis both alone and in conjunction with AmB. Initial experience was in 30 patients with candidal UTIs, almost two-thirds of whom (63%) had positive cultures at other sites.[91] The drug was administered for 4–6 weeks at a dosage of 100 mg/kg/day. *Candida* was eradicated from urine and other body sites in 77% of patients. Of the seven patients whose infection persisted, four appeared to improve clinically but continued to have significant colony counts in the urine. Two other patients had counts above 100,000/ml and were subsequently treated with intravenous AmB. The authors did not distinguish whether the species treated were *C. albicans* or non-*albicans*.

In a large prospective trial, 225 patients with candiduria initially received FC 100 mg/kg, with dosage adjustments based on serum levels.[94] The mean duration of therapy was 24 days, and a mean overall success rate of 94% was reported based on laboratory and clinical findings.[94] The most common adverse effect was diarrhea, which was reported in 10% of patients. Two cases of agranulocytosis resolved after discontinuation of FC.

A 14-day FC regimen of approximately 8 g/day was evaluated in 27 patients with fungal UTIs.[123] The overall success rate was 77.8%, with noncatheterized patients demonstrating highest clinical efficacy. From a microbiologic standpoint, FC was effective in eradicating 22 of 30 fungal strains. Various case reports have also described the drug's effectiveness in candidal UTIs (Table 4).[35, 58, 88]

Despite the reported success, resistance has been a major obstacle to FC's use. Approximately 7.6% of *C. albicans* are initially resistant to the agent (primary resistance).[124] In addition, resistance frequently develops during therapy, and the frequency appears to increase in patients with serum drug concentrations below 25 µg/ml.[118] Thus, some investigators have recommended that FC be dosed to obtain serum concentrations 4 times the maximum inhibitory concentration (MIC).[94] Due to lack of standardized susceptibility testing of fungi, however, the clinical significance of the MIC is unknown. Synergy with AmB has been demonstrated and may be a means to decrease toxicity as well as the development of resistance.[125]

Azoles

The azole class of antifungals consists of the imidazoles, ketoconazole, miconazole, and clotrimazole, and the triazoles fluconazole and itraconazole. Their mechanism of action involves disrupting fungal membrane permeability by inhibiting ergosterol synthesis. A summary of clinical studies with the imidazole agents is shown in Table 5.

Clotrimazole. Orally administered clotrimazole

Table 5. Clinical Studies of Imidazoles for Candidal Urinary Tract Infections

	No. of Pts.	Route	Duration (days)	Dosage (mg/kg/day)	Clinical Response	Comments
Ketoconazole	11	Oral	5–900	200–400	55%	Majority of failures were non-*albicans* sp.[127]
Ketoconazole	8	Oral	14	400	50%	3/4 failures had concomitant systemic infection.[126]
Miconazole	10	CBI	5	50	80%	Follow-up at 1–10 wks yielded no growth in patients who responded.[129]

CBI = continuous bladder irrigation.

Table 6. Clinical Studies of Fluconazole for Candidal Urinary Tract Infections

No. of Pts.	Route	Duration (days)	Dosage (mg/kg/day)	Clinical Response	Comments
7	Oral/i.v.		50	85.7%	Underlying conditions: bladder tumor, neurogenic bladder, hydronephrosis.[140]
6	Oral	30	50	100%	Urine cleared day 7 (2), day 15 (1), day 21 (3).[136]
7	Oral	7–56	50	71.4%	Failures were *C. albicans* (1), *C. tropicalis* (1).[135]
22	Oral/i.v.		50–100	86%[134]	
10	Oral/i.v.	72[a]	180[a]	100%	Patients had failed or could not tolerate previous fungal therapy.[139]
13	Oral/i.v.	> 7	50–100	92.3%	6 i.v., 1 p.o.; 1 failure in i.v. group.[132]

[a]Represents mean value.

resulted in resolution of candiduria in six of nine patients. However, serious adverse effects including gastrointestinal distress and mental status changes were noted in 50% of those who responded.[126] With the advent of safer and more efficacious imidazoles, clotrimazole is currently reserved for topical fungal infections.

Ketoconazole. Ketoconazole is primarily eliminated by hepatic metabolism, with excretion of only a small amount of active drug in the urine. The amount in the urine often does not reach concentrations higher than the MIC for the fungal organism, which limits the agent's use in candidal UTIs.

In a clinical study of eight patients with candiduria, ketoconazole 400 mg/day was administered for 2 weeks.[127] All patients had underlying diseases; six diabetes, one carcinoma of the bladder, and one a rejected kidney transplant. The drug failed to eradicate or reduce the urinary *Candida* counts in four patients, two of whom developed systemic fungal infection, and one who had a fungus ball.

Eleven patients, six with upper tract, five with lower tract infections, were given ketoconazole 200–400 mg/day.[127] *Candida* albicans was isolated from eight patients and *C. glabrata* (*T. glabrata*) from two, and one patient had both *C. tropicalis* and *C. glabrata*. Overall, *Candida* was eradicated in only 6 of the 11 patients. All those who did respond cleared their urine within 7 days except for one patient with nephrolithiasis, who required 180 days of therapy. Three of the five patients who failed therapy had a lower tract infection and three had infections caused by *T. glabrata*. The other failures were infected with *C. albicans* and *C. tropicalis*, with one case each.

In another report, a cure was observed in only one of three patients who received ketoconazole for fungal UTIs. The other two patients had received at least 100 days of therapy and were still being treated at the time of assessment.[128] Thus, available studies suggest, at best, a very limited role for the drug in the treatment of candidal UTIs.

Miconazole. Six patients received intravenous miconazole for the treatment of urinary candidiasis.[126] They all had concomitant systemic disease. Miconazole was effective in three patients, but two who were clinical successes received bladder irrigation of miconazole in combination with systemic therapy.

Miconazole was evaluated as CBI in 10 patients with candidal UTIs.[129] The drug was prepared in 1 L normal saline at a concentration of 50 µg/ml and administered continuously for 24 hours for a total of 5 days. The overall success rate was 80%, although one patient required a second course of therapy (at a miconazole concentration of 100 µg/ml) to eradicate the organism. The two who failed therapy had positive cultures at other sites that required systemic treatment with AmB. Despite its limited use, miconazole possesses several potential advantages over AmB, including stability when exposed to light, ease of preparation, and less expense, making it a plausible alternative.

Fluconazole. Fluconazole is a triazole antifungal with a mechanism of action similar to

ketoconazole's. It has a pharmacokinetic profile with potential advantages over other azoles. After oral administration it is well absorbed, with bioavailability greater than 90%, and unlike ketoconazole, absorption is not pH dependent.[130] Approximately 70% of a dose of fluconazole is excreted in urine as unchanged drug. Furthermore, its serum half-life of approximately 22–32 hours, allows for convenient once-daily dosing.[130]

Serum and urinary concentrations of fluconazole were examined in healthy volunteers.[131] After a single oral dose of 100 mg, the mean peak plasma concentration was 1.87 µg/ml 2 hours after dosing. Concomitant urinary data showed concentrations exceeding 30 µg/ml up to 12 hours after administration. Recovery of unchanged drug in the urine after 5 days was 75.1%. In theory, these pharmacokinetic data support the use of fluconazole in the treatment of candidal UTIs or cystitis, although the medical literature is sparse on this use.

To date, clinical studies with fluconazole for the treatment of candidal UTIs are summarized in Table 6. Fluconazole was evaluated in a multicenter study for the treatment of various fungal infections. Twelve (92.3%) of 13 patients with *Candida* UTIs were cured with dosages of 50–400 mg/day.[132] Of the six who received intravenous therapy, only one did not respond. All patients receiving oral therapy responded. The duration of therapy in all patients was at least 7 days. In other studies involving 38 patients with *Candida* UTIs, fluconazole cured 20 and led to improvement in 10, for an overall response rate of 79%.[133]

Clinical experience with the drug in patients with UTIs caused by both *C. albicans* and non-*albicans* sp has been described briefly. In clinical trials involving from 7 to 22 patients, response rates ranged from 71–100%.[134–137] The majority of patients received 50–100 mg/day for a range of 16–72 days. In one study, fluconazole successfully treated *T. glabrata* and *C. tropicalis* infections. However, another patient infected with *C. tropicalis* was noted to fail fluconazole therapy.

Two case reports demonstrated the agent's effectiveness in deep-seated renal infections. A renal abscess secondary to bacteremia and *C. albicans* was successfully treated with a 47-day course of fluconazole 50 mg/day.[138] A diabetic man with idiopathic renal papillary necrosis had numerous positive *Candida* cultures from midstream urine samples and from a renal papilla.[139] Because of the renal impairment, he received fluconazole 3 mg/kg every 96 hours. The treatment duration was 4 weeks, however, urine cultures were reported to be negative 4 days after the start of therapy. Although the patient relapsed, he again rapidly responded to fluconazole therapy.

The efficacy of fluconazole for treating complicated UTIs was evaluated in seven patients with underlying bladder or renal pathology.[140] *Candida* sp were isolated in all patients on at least two occasions in the week prior to therapy. Doses of 50 mg/day were administered orally to four patients and intravenously to three. The overall microbiologic response rate was 85.7%, with six of seven *Candida* sp eradicated. Clinical response was judged to be excellent in three patients, moderate in three, and poor in one.

Although the available data with the drug are promising, one area of concern is its use for treating infections caused by non-*albicans* sp. Failures of fluconazole against *T. glabrata* and *C. tropicalis* have been reported in open studies involving urinary tract and other deep-seated infections.[135, 138] In addition, *C. kruseii* is known to be inherently resistant to the agent.[141] Thus, caution should be exercised in using it for UTIs due to species other than *C. albicans* until further studies address this issue.

Fluconazole is generally well tolerated in dosages ranging from 50–400 mg/day. In clinical trials, adverse effects were reported in approximately 16% of patients,[142] most commonly, nausea, abdominal pain, headache, rash, diarrhea, and vomiting. Discontinuation of therapy was required in approximately 1.6% of patients.

Although the drug is not as potent an inhibitor of the cytochrome P-450 system as ketoconazole, several drug interactions have been identified. Concomitant administration of fluconazole with tolbutamide, glyburide, and glipizide demonstrated increases in area under the curve and maximum concentrations of the oral hypoglycemics, leading to clinically significant hypoglycemia in some patients.[142] Fluconazole also increased serum concentrations of phenytoin and cyclosporine, as well as potentiating the anticoagulant effect of warfarin.[142]

Recommendations

The decision to treat or not to treat a candidal UTI is often based on clinician preference and experience, as well as evaluation of the individual patient. Severity of illness, risk factors, and practical considerations all must be assessed. There are few data concerning the natural progression of the disease, and studies that are available indicate these infections rarely contribute to patient mortality. They also suggest that candidal UTIs persist in the majority of patients unless risk factors are minimized. If *Candida* is recovered from the urine in a quantity greater than 10^3 cfu/ml, the first mode of therapy should be to minimize risk factors, including removing or replacing a Foley catheter. If this is not possible or is ineffective, antifungal therapy should be considered.

Local instillation of AmB should be considered the treatment of choice for most patients due to its demonstrated efficacy, safety, and short duration. The standard dose is 50 mg in 1 L sterile water given as a continuous bladder irrigation for 5 days. When catheterization is not possible, fluconazole may be given as initial therapy. Most studies used dosages of 50–100 mg for at least 7 days. Reculture should be performed after 7 days of therapy to determine if longer treatment is needed. Additional data concerning dose and duration of therapy are necessary before more widespread use can be recommended. In addition, caution should be exercised in treating UTIs caused by organisms other than *C. albicans* due to fluconazole's questionable activity against non-*albicans* sp. Although the results of two very small studies were promising, there is not enough experience with single-dose AmB to recommend it. Although FC is effective, resistance and toxicities limit its use; the agent has generally fallen out of favor with the advent of fluconazole. The imidazoles are not indicated in the treatment of candidal UTIs due to toxicity and poor response rates.

Summary

Fungi, especially *C. albicans*, are becoming increasingly important nosocomial pathogens. The isolation of *Candida* has increased at all major sites of infection including the urinary tract. Despite its increased prevalence, the significance of candiduria remains uncertain. Risk factors and pathogenesis have been fairly well characterized, but questions concerning diagnosis and treatment remain. In fact, it is currently unknown if candiduria requires treatment.

Patient-specific factors must be considered in the decision to treat. When treatment has been instituted, AmB CBI has been the standard. Although it is associated with high success rates, the frequency of relapses must be addressed. The role of the new triazoles in the treatment of candidal UTIs must be established through well controlled clinical trials. These agents are promising, but questions remain concerning their spectrum of activity, dosing, and optimum duration of therapy.

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Exhibit C

MOLECULAR METHODS FOR EPIDEMIOLOGICAL AND DIAGNOSTIC STUDIES OF FUNGAL INFECTIONS

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Summary

Over the past two decades there has been a remarkable increase in the incidence of invasive fungal infections. Molecular methods, such as karyotyping, restriction analysis and polymerase chain reaction (PCR), have now been applied to improve our current understanding of the epidemiology of these fungal infections. For example, investigations on nosocomial outbreaks of fungal infections have been greatly facilitated by molecular methods. In addition, the ability to diagnose and identify deep-seated mycoses may be enhanced by the use of molecular techniques. In the near future it is possible that PCR-based methods will supplement, or perhaps even replace, traditional methods for detection of *Candida albicans* blood stream infections, invasive aspergillosis and *Pneumocystis carinii* pneumonia. This review examines the progress of molecular biology into the clinical arena of fungal epidemiology, laboratory identification and diagnosis.

Key words: Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans, diagnosis, DNA fingerprinting, DNA probes, epidemiology, fungal infections, molecular biology, PCR fingerprinting, polymerase chain reaction, random amplification of polymorphic DNA restriction enzyme analysis, restriction length fragment polymorphism.

Abbreviations: BAL, bronchoalveolar lavage; CHEF, contour-clamped homogeneous electric field electrophoresis; MEE, multilocus enzyme electrophoresis; PCP, *Pneumocystitis carinii* pneumonia; PFGE, pulsed-field gel electrophoresis; RAPD, random amplification of polymorphic DNA; REA, restriction enzyme analysis; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformational polymorphism.

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INTRODUCTION

Fungal infections have increased in incidence over the last two decades due to increasing prevalence of immunosuppressed patients and medical advances such as the use of potent broad spectrum antibacterial agents. For instance, in patients undergoing solid organ and bone marrow transplantation, invasive fungal infections are a major obstacle in treatment success.[1,2] Severe oropharyngeal candidiasis and cryptococcal meningitis were relatively rare entities before the AIDS epidemic, but they are now common clinical problems. Fungi, once considered to be microbiological curiosities without pathogenic potential, have emerged as opportunistic human pathogens[3] since their immunosuppressed hosts live longer. Also, multiple cases of possible nosocomial outbreaks of fungal infections have been published in the past five to ten years. These reports emphasize the need for understanding the epidemiology of fungal transmission.

In parallel with the epidemic of fungal infections, the medical community has also witnessed revolutionary progress in the field of molecular biology. The rapidly expanding field of medical mycology continues to benefit from this scientific progress. In studies on fungal phylogeny, new DNA-based methods have been established which allow relationships between fungi to be understood and which identify and classify new fungi such as *Pneumocystis carinii*.[4] Recently there have been significant advances in the use of molecular biology to study fungal virulence factors and pathogenesis.[5] The ability to transfer DNA into cells, produce gene disruptions and replacements and examine regulation and signalling pathways through their genes has also been improved.

The principal methods for epidemiological purposes in clinical molecular mycology include restriction enzyme analysis (REA), DNA hybridization analysis (Southern blotting with probing of DNA), karyotyping with pulsed-field gel electrophoresis, and the use of the polymerase chain reaction (PCR) for amplification of random polymorphic DNA fragments. Although by no means obsolete, the traditional mycological methods of culture, serology and morphological studies can now be enriched with these novel molecular applications.[6] For example, PCR technology with fungal-specific primers and fungal-type specific probes are now available, and with further commercial development they could be used for diagnostic purposes on clinical specimens. These methods have the potential to improve diagnostic accuracy and hasten the institution of specific antifungal therapy. In this review we will summarize some of the recent advances in molecular mycological epidemiology and diagnosis of fungal infections.

EPIDEMIOLOGY

Most serious fungal infections affect those patients already immunocompromised. For example, *Candida albicans* is a common pathogen in hospitalized burn patients, low-birth-weight infants, recipients of parenteral nutrition and patients in intensive-care units on broad-spectrum antibacterial agents.[7] *Cryptococcus neoformans* is a major pathogen in AIDS patients, and infected patients require life-long antifungal suppression since relapses of infection are common.[8] *Aspergillus fumigatus* can cause invasive infections in neutropenic and bone-marrow transplant patients[2] and has an extremely high rate of mortality.[9]

There is always a concern about environmental expo-

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TABLE 1 A summary of molecular mycological methods used in epidemiological studies, with advantages and disadvantages of each method

Method	Advantages	Disadvantages
REA	Simple Reproducible Available in many clinical laboratories	Poor resolution of bands Low to moderate discrimination of strains
RFLPs and hybridization	Reproducible High discrimination of strains	Laborious Time-consuming
Karyotyping (PFGE)	Simple Available in some clinical laboratories	Time-consuming Results variable in some organisms Needs criteria for distinguishing differences
RAPD	Simple High level of strain discrimination	Poorly standardised; needs careful quality controls Problems with reproducibility Currently only available in research laboratories
Repetitive sequence-based PCR (PCR fingerprinting)	Simple Reproducible Excellent level of strain discrimination	Currently only available in research laboratories

sures for acquisition of mould infections. Thus, the ability to type fungal strains by using molecular methods is important in investigations of possible nosocomial outbreaks of fungal infections and for determining relapses from reinfections with certain clinical isolates. Molecular methods have been used to study relationships between isolates of candidosis and aspergillosis, with the goal of distinguishing isolates, thus making it possible to track the sources of infections and hence to identify strategies for improving preventive measures. These methods are listed in Table 1 together with their advantages and disadvantages.

Restriction enzyme analysis (REA)

The term restriction fragment length polymorphism (RFLP) is commonly used to describe specific band patterns of genomic DNA that can be directly visualized on a gel after digesting DNA with a variety of restriction enzymes.[10–12] This methodology is also referred to as REA. Different species and strains may have different band patterns due to polymorphic variations in their DNA sequences that change the location of the restriction enzyme sites. There are several clinical studies which have demonstrated the utility of RFLPs for epidemiological studies on *C. albicans*[13,14], *Candida inconspicua*,[15] *Candida glabrata*[16] and *Saccharomyces cerevisiae*.[17] This methodology is most commonly used in investigations on nosocomial outbreaks of fungal infections. For example, Bart-Delabesse and co-workers studied 20 azole-resistant *C. albicans* and *Candida parapsilosis* isolates using restriction analysis, and found that some of the strains were transmitted from a burn unit nursing staff member to patients.[14] Using RFLPs from two restriction enzymes an outbreak of *Candida tropicalis* sternal-wound infections was linked to a scrub nurse.[18] A pseudoepidemic of *C. parapsilosis* due to presence of the fungus in a laboratory solution was identified by showing identical RFLP patterns.[19] By careful selection of restriction enzymes, this method can, in some instances, be superior to other genotypic methods such as karyotyping of *C. albicans*.[20]

An improved understanding of the environmental source of clinical isolates of *A. fumigatus* by the use of RFLP analysis could facilitate preventive strategies.[21] Lin and colleagues performed RFLPs on 35 clinical and environmental isolates of *Aspergillus* and found that 22 unique strain patterns could be identified by combining the results from two restriction enzymes and visualizing the bands directly on a gel.[22] The marked increase in coccidioidomycosis in California in 1992, led one group of investigators to study *Coccidioides immitis* by RFLP analysis. Two major genotypic groups were identified, but no association with virulence was demonstrated in either group[23]. The study of sporotrichosis has also been facilitated by restriction analysis. A case of zoonotic transmission of sporotrichosis has been confirmed at the genotype level by comparing RFLPs.[24]

Despite its reasonable discriminatory power, a potential problem of the REA/RFLP method has been the subjectivity demonstrated in the interpretation of complex band patterns. Uncut and/or partially digested DNA can cause background which may also be confounding. This can be circumvented by using appropriate band analysis software, which is commercially available. Alternatively, the discriminatory power can be increased by using Southern transfer of the DNA and hybridization with specific probes (see below).

DNA hybridization analysis

As outlined above, specific band patterns can be visualized after digesting DNA with restriction enzymes and separating it on a gel. This methodology can be extended by transferring the DNA to nitrocellulose or nylon membranes (Southern blotting) which can then be hybridized with specific probes.[25,26] Several probes have been designed with both fungal-specificity, based on highly conserved areas in the fungal genomes, as well as species-specificity.

used to differentiate species within a genus.[27–29] DNA probes can be broadly classified into three categories: (a) repetitive DNA probes derived from the organism under study; (b) minisatellite probes; (c) synthetic oligonucleotides which are complementary to repetitive sequences in the fungi.

The moderately repetitive sequence, Ca3, has been shown to be a very effective probe for discriminating between strains of *C. albicans*.[30–33] Other probes specific for *Candida krusei* and *C. tropicalis* have been described.[34–37] Probing with repetitive sequences has also proven useful in epidemiological studies on *C. neoformans*.[38–41] As an example, the dispersed repetitive sequence, CNRE-1, in *C. neoformans* has been used in the study of pathogenesis and in epidemiological investigations of cryptococcosis.[41–43] The epidemiological study of other fungi such as *A. fumigatus*,[21,22,44–47] *Aspergillus flavus*,[48,49] *Histoplasma capsulatum*[50] and *Blastomyces dermatitidis*,[51] has also benefited from this particular methodology.

There are a series of clinical studies which demonstrate the utility of using RFLPs and DNA probing for epidemiological studies of *C. albicans*.[13,20,52–56] For instance, multiple *Candida* strains isolated during the course of a single infection can be identified using the Ca3 probe.[53] The use of this probe has revealed molecular similarities between hospital isolates of *C. albicans*, providing evidence for nosocomial transmission.[55] In addition, in an attempt to identify specific physiological traits that are associated with colonization and pathogenesis, the Ca3 probe has recently been utilized to identify and classify similar strains that are more frequently isolated from patients suffering from candidosis.[54] A recent study by Pujol and colleagues, where the discriminatory ability of RFLPs and probing with Ca3 was compared to the randomly amplified polymorphic DNA (RAPD) method and multilocus enzyme electrophoresis (MEE), showed that all methods were similarly effective.[33] By using the Ca3 probe or its C1 fragment, it has also been shown that colonizing populations of *C. albicans* are generally clonal in origin but can subsequently undergo microevolution *in vivo*.[33,56] Similarly, by using three different probes Lockhart *et al.* showed that recurrent *C. albicans* vulvovaginitis is due to strain maintenance of the same strain in the majority of cases, but formation of different substrains in a random fashion, a process referred to as 'substrain shuffling', also occurs in a third of the cases.[57] Atypical *Candida* isolates taken from HIV-infected individuals have been analyzed by using the 27A repetitive sequence, demonstrating genetic characteristics identical to those of *Candida dubliniensis* and suggesting widespread geographic distribution of this novel species.[58] The same 27A probe has also been shown to yield a higher degree of strain discrimination in *C. albicans* than karyotyping, *Not*I restriction analysis or PCR fingerprinting.[52]

The epidemiology of *C. neoformans* infections remains poorly defined and the natural reservoir for human infection is unknown for var. *neoformans* (serotypes A and D). Pigeon excreta have, however, been suggested as potential sources for infection. Supportive evidence for this theory has come from studies in New York and France, where comparisons of clinical and environmental isolates revealed that patient isolates had similar RFLP patterns to those found in the pigeon droppings.[41,59] On the other hand, patients do not seem to be disproportionately infected by particular strains since another study did not reveal a specific pattern for strains taken from AIDS patients versus non-AIDS patients or from the environment.[60] By comparing RFLP patterns of serial isolates and probing with the repetitive probe CNRE-1, Spitzer and colleagues showed that recurrences of cryptococcal meningitis generally result from persistence of the original infecting strain rather than acquisition of a new strain.[61] Similarly, the CNRE-1 probe has been used to demonstrate that persistent cryptococcal infection in AIDS patients is generally due to relapse rather than reinfection.[62] By using another repetitive probe, CND 1.7, it has been shown that the isolates of *C. neoformans* in patients with and without AIDS are similar.[40] Finally, another repetitive probe for *C. neoformans*, UT-4p, has been shown to be highly discriminatory between strains.[59,63]

Minimal DNA variability between *A. fumigatus* isolates was found by using RFLP analysis and probing with the *Aspergillus nidulans* ribosomal repeat unit.[64] In contrast, two studies using a moderately repeated DNA sequence as a probe to fingerprint *A. fumigatus* from patients with invasive aspergillosis, have shown that most clinical isolates exhibit different hybridization patterns and that individual patients were usually infected by a single strain.[44,45,65] In addition, a temporal survey of environmental isolates showed that some *Aspergillus* strains can persist in the same environment for at least six months.[44] DNA typing of *A. fumigatus* by RFLPs can therefore distinguish isolates from different origins and clinical situations.[21] Specific DNA probes have also been constructed for epidemiological identification of other *Aspergillus* species, such as *A. flavus*.[49]

Histoplasma capsulatum has been divided into three classes by polymorphisms within its rDNA.[50,66] Further studies, using a specific nuclear probe, have revealed six broad genotypic classes, which appear to reflect the geographic origin of the isolates.[67] These studies have also provided molecular support for the notion that AIDS-associated histoplasmosis in nonendemic areas is due to reactivation of a previously acquired infection from another environmental site.[67]

Other less common fungal pathogens are being studied with increasing intensity using these molecular methods. Clinical isolates of *Sporothrix schenckii* from an epidemic in 1988, have been shown to be genetically indistinguishable from environmental isolates using DNA hybridization analysis.[68] When fungi are in early molecular development, probes from other species (heterologous probes) are commonly used for molecular typing. For instance, by using a mitochondrial probe from *H. capsulatum*, three classes of RFLPs have been identified among 19 clinical isolates of *Blastomyces dermatitidis*.[51]

Karyotyping

While the utility of REA and DNA hybridization analysis is well established, several alternative methods have been developed that may be complementary and perhaps even more useful in the clinical arena. Karyotyping, or separation of fungal chromosomes on a gel, can be used in studies on fungal epidemiology, since chromosome polymorphisms are a common feature of many pathogenic fungi. The chromosomal size variability can be studied

using pulsed-field gel electrophoresis (PFGE), most often performed with contour-clamped homogenous electric field electrophoresis (CHEF). Comparative studies on the different methods for typing of *Candida* sp. have already been published.[20,69–71] However, further comparisons of karyotyping to other methods need to be performed focusing not only on other genera and differences in discriminatory power, but also on time needed and cost involved in these procedures.

Karyotyping and DNA fingerprinting have been suggested to have the highest discriminatory power among genetically related *Candida* isolates.[14,30,71] Until recently it was not known whether changes in karyotype of *C. albicans* isolates during the course of infection in neutropenic patients, were due to rearrangements or infection with new organisms. Serial karyotypic analysis of *C. albicans* isolates in the same patient has shown that changes in karyotype can indicate infection by a new strain.[14,72] The usefulness of karyotyping for other *Candida* species has also recently been documented in epidemiological investigations of *Candida rugosa*, an emerging pathogen in burn units,[73] and *C. parapsilosis*.[74] Dib and colleagues analyzed 9 *C. rugosa* isolates from burn victims using karyotyping as well as REA. They demonstrated the same patterns among the outbreak isolates, strongly suggesting clonal strain transmission.[70] In addition, the karyotyping method seemed to have better discriminatory power than REA for diverse unrelated control strains.[70] Karyotyping has also been recommended over isoenzyme profile analysis for epidemiological investigations with *C. tropicalis*.[75] Furthermore, karyotyping has been shown to be superior to REA analysis in discrimination of some clinical isolates of *C. glabrata* and other non-*albicans Candida* species.[69]

Karyotyping of *C. neoformans* (Fig. 1) has also been shown to be a feasible method for the study of infections by this yeast, since significant strain polymorphisms have been found. The number of *C. neoformans* chromosomes can vary between seven and 13.[76] For example, a study of 40 clinical and environmental isolates showed that 90% of the strains exhibited unique chromosome banding patterns by PFGE.[77] In addition, the karyotypes with one strain remained stable during passage both *in vitro* and *in vivo*. It has therefore been proposed that the primary use of this method may be for epidemiological studies and to distinguish between strain relapses and reinfections.[77] Barchiesi and co-workers used this technique to analyze serial isolates from the same patients. They demonstrated that the electrophoretic karyotype changed in some of the patients during treatment, suggesting mixed infection, whereas in others the profiles remained unchanged during treatment.[78] Kwon-Chung and colleagues showed no common karyotype in clinical isolates of *C. neoformans* var. *gattii*, whereas all strains isolated from eucalyptus trees had an identical karyotype.[79] Further investigations by Fries *et al.* have confirmed extensive variation among isolates from different patients.[80] On the other hand, in this study sequential isolates from the same patients showed minor differences in electrophoretic karyotypes in up to 50% of the patients. This finding suggests that chromosomal rearrangements or deletions can occur in some strains during infection in the host.[80] Furthermore, these chromosomal changes have been replicated in animal models and demonstrate the plasticity of the *C. neoformans* genome. Therefore, minor chromosomal variations in karyotypes should be interpreted with caution in this organism. Thus, when there are minor changes in karyotypes of sequential isolates, strict criteria for significant differences to identify unique strains may be required, such as at least two band differences in the karyotype and a second molecular typing method.

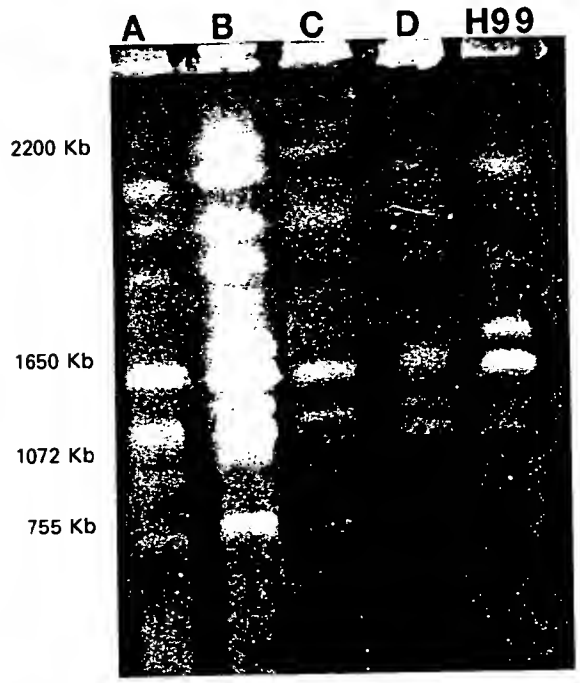

Fig. 1 Karyotype gel demonstrating the chromosone patterns from *Cryptococcus neoformans*. The yeast strains used (A–D and H99) were five clinical serotype A strains.

The chromosomes of *Pneumocystis carinii* have been studied by PFGE, revealing 17–19 chromosomes and, unlike *C. neoformans*, a relatively stable karyotype band pattern that is maintained over time in isolates from a single host.[81] Moreover, different strains and mixed infections can be determined by PFGE patterns with *P. carinii*.

Other fungi with fewer chromosomes than the seven to eight in *C. albicans*,[82–84] or the seven to 13 observed in *C. neoformans*[77], may be less ideal for use of karyotyping as a molecular typing system, since a low number of chromosomes decreases the discriminatory power of the method. For example, *Paracoccidioides brasiliensis* has four chromosomes[85] and *C. immitis* only has three large chromosomes[86].

PCR-based methods

Although RFLPs and karyotyping can be powerful tools in epidemiological studies, the complexity and time-intensity of these procedures may preclude their use in a clinical or even research microbiological laboratory. Recently simple PCR-based methods have been introduced which may provide more practical tools in clinical epidemiology.

One of the advantages of PCR-based methods is their extreme sensitivity. By using these methods we are able to analyze minimal amounts of DNA in the environment as well as in patient samples. Furthermore, the development of PCR has revolutionized our ability to study unculturable pathogens. As an example, our current understanding of

the epidemiology of *Pneumocystis carinii* pneumonia has benefited greatly from this progress. By filtering air from diverse locations and performing PCR on the DNA trapped in the filters, Bartlett *et al.* were able to show that *P. carinii* DNA was present in 57% of air samples from the rooms of *P. carinii*-infected patients and 29% of other hospital rooms, whereas it was not found in any of the control sites.[87] Thus, these data contradict our previous theories of the epidemiology of this infection and suggest that the disease could be transmissible among patients.

Random amplification of polymorphic DNA (RAPD): Over the past five years the use of arbitrary nucleotide sequences (primers) to amplify genomic DNA sequences, or RAPD, has been intensively studied in fungi. This technique was first described by Welsh and McClelland[88] and Williams and co-workers.[89] Several different terms have been coined to describe this technology, including arbitrarily primed PCR[88], RAPD[89] and DNA amplification fingerprinting.[90] The general strategy is to observe genomic differences in fungal strains from the same species.[91] The RAPD method is less time-consuming than RFLP analysis and is ideally suited for rapid screening of a large number of isolates. However, a basic warning to all new investigators with the use of this testing strategy is that careful quality controls and conditions must be set for reproducibility prior to performing the actual study. If not adequately controlled, the reproduction of band patterns may be difficult (Table 1).

For *C. albicans*, RAPDs have been shown to provide a fast, economical and reasonably reproducible method for molecular typing.[92] Some authors have shown that the level of discrimination by this method approaches that of karyotyping.[71] Furthermore, more recent studies suggest that both restriction analysis and RAPD may even surpass karyotyping in discriminatory power.[20] An example of the usefulness of this methodology was given by Robert *et al.*, who demonstrated in a prospective study that 68% of all *C. albicans* isolates in their burn unit had identical profiles, which suggested that nosocomial acquisition of certain strains had occurred in this setting.[93] Dahl and co-workers used RAPD to demonstrate that oropharyngeal and vaginal *C. albicans* isolates from HIV-positive women were dissimilar, suggesting that the dominant strain of *Candida* colonizing different body sites is also different.[94] Furthermore, the method was shown to be of similar sensitivity as RFLP analysis with DNA probes. A potential disadvantage of RAPD, however, is the lower reproducibility of the method.[33]

Epidemiological studies on *C. neoformans* have been performed by using RAPD and have found that both clinical and environmental isolates belong to the same pool of isolates.[95] Further studies show that multiple isolates from the same patient always have the same RAPD patterns.[38,96,97] In some patients with AIDS and cryptococcosis, it has been shown, however, that multiple strains may be involved in a single infection and, at the very least that strains can evolve during infections and be replaced by new genotypes during recurrent infections.[39,98] These findings support the notion that *C. neoformans* may undergo microevolution within their host, akin to the observations on karyotypic instability noted by Fries and co-workers.[80] In a large survey, MEE and RAPD were evaluated by Brandt *et al.* for their usefulness in subtyping 344 clinical isolates of *C. neoformans*. In some isolates, RAPD improved the discriminatory power of MEE, suggesting that this method might be useful in ascertaining environmental sources of these infections.[99] Interestingly, in one study a particular RAPD profile was found to be highly represented in patients with AIDS and other immunosuppressive conditions. This finding suggested that the amplicons generated by this RAPD might contain sequences linked to a specific factor of virulence.[96]

Genotyping of *A. fumigatus* by RAPD has been successfully used to distinguish between clinical isolates.[100,101] An apparent outbreak of nosocomial aspergillosis in a hematology ward in The Netherlands was studied by using the RAPD technique. In this outbreak it was demonstrated that the strains were unrelated and apparently not due to a common source within the hospital.[102] Similarly, patients with cystic fibrosis (CF) have several genotypes of *A. fumigatus* in their sputum, as demonstrated by RAPD.[103] On the other hand, there are non-CF patients who reportedly have been colonized and/or infected by a single strain.[44,65] Anderson and colleagues studied 16 selected isolates of *A. fumigatus* by RAPD and RFLPs and probing with the total genome of the bacteriophage M13.[104] They found that a combination of the two methods gave the highest level of discrimination. In another study, RAPD was found to have superior discriminatory power to RFLP and repetitive probes in genotyping of *A. fumigatus* isolates; RAPD classified 61 strains into 54 groups, whereas RFLP analysis of the mitochondrial DNA and rRNA genes revealed no variability.[64]

For other fungi, such as *H. capsulatum*, RAPD testing allows isolates to be distinguished in a more sensitive manner than has been possible by conventional RFLP analysis.[105,106] For example, Kersulyte and colleagues were able to discriminate between each of 29 clinical isolates within a single RFLP class by use of only three arbitrary primers.[106] Similarly, Yates-Siilata and colleagues compared REA to RAPD in 19 clinical isolates of *B. dermatitidis*. Whereas three major classes were identified by using restriction analysis, dissimilar RAPD patterns were observed within the major classes. The RAPD methodology can thus improve strain discrimination and, if reproducible, may be a better tool for epidemiological studies than present REA.[51]

The epidemiology of the dematiaceous hyphomycete *Scedosporium prolificans*, which causes focally invasive infections in immunocompetent individuals and invasive infections in immunocompromised patients, has recently been studied by RAPD. Millán and colleagues showed that all 17 different isolates from patients in Spain could be discriminated by the method, whereas sequential isolates from the same patients were identical.[107] A RAPD study on 11 isolates of another dematiaceous fungus, *Exophiala (Wangiella) dermatitidis* from patients with CF, revealed four different genotypes.[108] Furthermore, RAPD has been used to demonstrate that the zygomycete *Mucor indicus*, isolated from a bone-marrow transplant recipient with hepatic mucormycosis, was actually acquired by ingestion of a naturopathic medicine containing a genetically identical organism.[109]

PCR using repetitive sequences as primers (PCR fingerprinting): Simple repetitive DNA (microsatellite and minisatellite) sequences in the fungal genome can be used as oligonucleotide probes for conventional Southern blots. The same oligonucleotides can also be used as single primers with PCR to generate individual band patterns (also called PCR fingerprinting). Microsatellite DNA may provide a general source of molecular markers to study epidemiology and evolution of microorganisms.[110] Repetitive sequence-based PCR may be more reliable than other PCR-based techniques for the detection of polymorphic DNA, since the primers are longer and because of strong homology between the primers and the target sequences.[111] The advantage of this methodology is its fairly high discriminatory power together with the high reproducibility and the stability of the markers.

Microsatellite and minisatellite polymorphism in *C. albicans* has been shown to be easily detected by PCR,[112,113] and to be reproducible, although intensity of the bands may vary.[114] This technique has been used to differentiate between several isolates of *C. albicans* in neutropenic patients. These results suggest usefulness in patient monitoring and studies on clonality.[115] Other species of *Candida*, including *C. tropicalis*,[116] *C. krusei* and *C. rugosa*, are also easily distinguished by this method.[117]

Repetitive sequence-based PCR has been evaluated in *Cryptococcus neoformans* (Fig. 2) and confirmed for its feasibility in taxonomical, epidemiological and diagnostic studies.[111,118] In a large epidemiological study, 110 environmental and clinical isolates of *C. neoformans* var. *gattii* were analyzed by RAPD and PCR fingerprinting, and shown to contain three different major genetic profiles.[119] It was further shown that a single profile was predominantly found in clinical isolates as well as isolates from eucalyptus trees, thus supporting the hypothesis that human disease is acquired from direct exposure to *C. neoformans* var. *gattii* on eucalyptus trees.

Genotyping of *Aspergillus* species by PCR of repetitive DNA motifs revealed that the method was only powerful enough to discriminate between *Aspergillus* strains at the species level. On the other hand, when a primer derived from a prokaryotic repeat motif was used, different *A. fumigatus* isolates could then be typed individually.[120]

Importantly, apart from their use in epidemiology, microsatellite polymorphisms can be studied using automated procedures, as has been done in human genetics[112], which will facilitate the construction of genetic maps of fungi in the future.

PCR with single-strand conformational polymorphism (SSCP): A novel method, termed single-strand conformational polymorphism (SSCP), utilizes conformational polymorphism of the amplified PCR products which results from minor sequence variation in the DNA, to distinguish fungal genera and species.[121] Walsh and colleagues used primers specific for the 18S rRNA gene to amplify a 197-bp fragment common to all medically important fungi. The fragment was denatured and run on a gel and subsequently it was shown that the SSCP banding patterns were unique to all of the species analyzed, including *Aspergillus*, *Candida* and *Cryptococcus*[121]. This methodology has also been used in population studies on *C. albicans*, *C. immitis* and *H. capsulatum*[122–124]. Due to its potential for high discriminatory power at certain genetic loci, it seems to hold promise for further epidemiological and taxonomic studies within research laboratories.

Direct sequencing

Direct sequencing of PCR products is now a widely used tool in molecular biology. The ability to circumvent laborious and time-consuming cloning procedures as well as the development of automated sequencers makes this methodology attractive in epidemiological studies on fungal infections. Given the inability to culture *Pneumocystis carinii in vitro*, molecular methods are particularly well suited for studies on this organism. Two studies have have focused on bronchoalveolar lavage (BAL) and induced sputum samples from HIV-infected patients who have had one or several episodes of *Pneumocystis carinii* pneumonia (PCP). PCR amplification and direct sequencing of the two internal transcribed spacers of its rRNA genes (ITS1 and ITS2) was used to distinguish relapse from reinfection in these patients.[125,126] Combined data from these two studies show that 19 of the 31 patients were infected by the same strain, whereas in 12 patients recurrence was due to *de novo* infection with a genetically distinct strain of *P. carinii*.[125,126]. These epidemiological findings may be of importance for further studies in implementing preventive measures.

DIAGNOSIS

Apart from the use of molecular methods in epidemiological and taxonomic studies, these powerful methods are also reforming the diagnostic process of fungal infections. Several fungal-specific probes as well as PCR-based assays are being developed to facilitate the diagnosis of fungal infections. The greatest importance of these methods is obviously for hospitalized and/or immunocompromised patients for whom diagnostic delay can be costly.[9,127,128]

Probes for identification of strains in clinical laboratories

For immunosuppressed patients, rapid identification of fungi in clinical specimens can be of paramount importance so as to clarify diagnosis and facilitate treatment decisions. DNA probes are now commercially available and have been shown to aid in the identification of fungi.[79,129–131] Gen-Probe's chemiluminescence-labeled DNA probes are complementary to fungal rRNA sequences.[132] It has been shown that the use of these probes can significantly reduce the time required for fungal identification. For example, Huffnagle and Gander were able to show that AccuProbe product for *H. capsulatum* and *C. neoformans* were both 100% sensitive and specific after testing 95 clinical mould phase isolates and 98 yeast isolates, respectively.[132] Similarly, by testing 1,149 different isolates, Stockman and co-workers evaluated the AccuProbes for their ability to correctly identify *B. dermatitidis*, *C. immitis*, *H. capsulatum* and *C. neoformans*.[133] Apart from the *B. dermatitidis* probe, which had a sensitivity of 87.8% to 97.3%, all probes were extremely sensitive (97.0 to 100%). In addition the specificity of all probes was 100% for all four fungi.

Compared to other commercially available methods, the short time (two hours) needed to perform the tests is an important advantage.[132] However, the major disadvantage

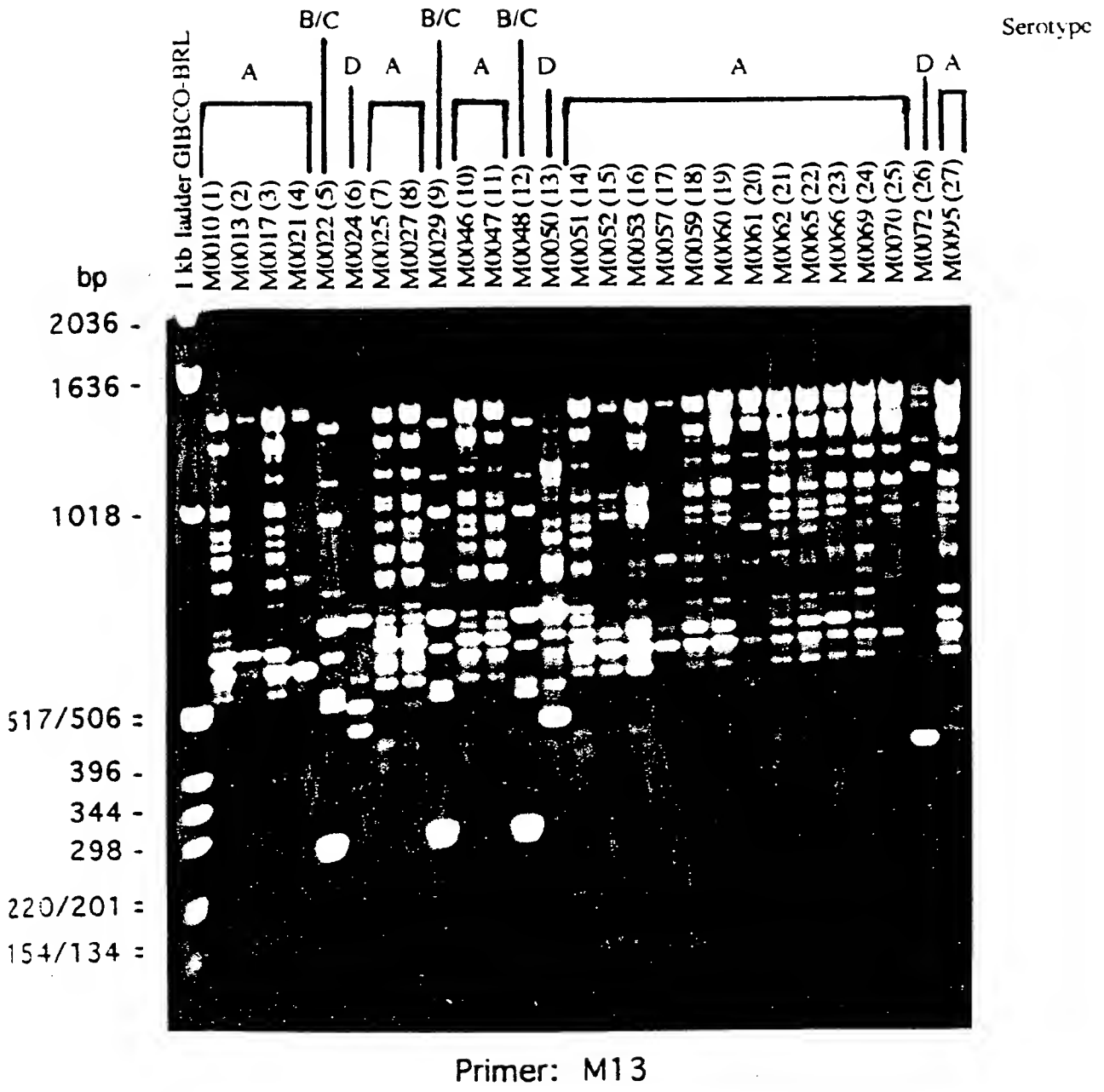

Fig. 2 PCR fingerprints of clinical isolates of *Cryptococcus neoformans*. Genomic DNA was amplified with the M13 core (minisatellite) sequence as a single primer. To identify serotypes, PCR band patterns were compared to reference strains of *C. neoformans* (serotypes A–D) and the results are indicated by the capital letters above the brackets. Courtesy of Dr W. Meyer. Reproduced from[111] with the publisher's permission.

of the AccuProbe are the requirement for expensive instrumentation and a probe shelf-life of only six months, which may be a drawback especially in small clinical laboratories.[132] These tests are probably not needed for some yeasts, such as *C. neoformans*, which grow rapidly and for which presumptive identification can be done quickly with existing methods.

Another commercially available test was evaluated in the clinical setting by Ferris and colleagues, who compared the traditional diagnostic methods (wet mount, KOH prep, amine and pH tests) to DNA hybridization, using specific probes for *Candida* sp., *Trichomonas vaginalis* and *Gardnerella vaginalis* in 501 women with vaginitis.[134] The sensitivity and specificity of clinician microscopically diagnosed vulvovaginal candidiasis were 39.6% and 90.4%, respectively, whereas the sensitivity and specificity of the DNA probe method were 75.0% and 95.7%. Similarly, the sensitivity of the clinical diagnoses of trichomoniasis and

TABLE 2 Human studies on the use of PCR for diagnosing invasive aspergillosis, listed by patient population, primers used for the PCR, sampling method, sensitivity and specificity

Organism	Patients	Primers	Samples	Prospective?	Sensitivity[1] (%)	Number[2]	Specificity[3] (%)	Number[4]	Ref.
A. fumigatus	Various	26S rRNA/ISR[5]	BAL/ BW/ TB[6]	No	67	4/6	76	13/17	140
A. fumigatus	Renal and bone marrow transplant	AP gene[7]	BAL	No	100	4/4	94	18/19	142
Aspergillus sp	Leukemia	18S rRNA[8]	BAL	No	100	6/6	63	5/8[9]	141
Aspergillus sp	Immunosuppressed	Mitochondrial DNA	BAL	No	75	3/4	75	36/48	144
Aspergillus sp	Hematological	18S rRNA	BAL	No	100	3/3	69	11/16[10]	139
Aspergillus sp	Various	18S rRNA	Serum	No	70	14/20	100	20/20[11]	143

[1] Sensitivity, calculated from numbers in article
[2] Actual number of subjects in study with a positive PCR result/total number of patients with proven disease as determined by culture, histology or cytology.
[3] Specificity, calculated from number in article.
[4] Actual number of patients in study with a negative PCR result/total number of patients with negative culture, histology or cytology.
[5] 26S rRNA/ISR: 26S ribosomal RNA gene/intergenic spacer region.
[6] BAL, bronchoalveolar lavage; BW, bronchial washings; TB, trap bronchoscopy.
[7] AP gene, alkaline phosphatase gene.
[8] 18S rRNA, 18S ribosomal RNA gene.
[9] Specificity is higher if non-immunosuppressed patients are included, 86% (19/22).
[10] Specificity is higher if non-immunosuppressed patients are included, 80% (41/51).
[11] Specificity calculations only based on PCR results from healthy volunteers.

bacterial vaginosis were lower than those of the DNA probe test.[134]

Recently Einsele *et al.* developed a PCR assay which is followed by probe hybridization to detect *Candida* and *Aspergillus* species in blood. They evaluated the method in 601 blood samples, demonstrating low detection limits (1 cfu/ml of blood) as well as sensitivity (100% if two specimens were tested).[135] Direct fluorescence *in situ* hybridization of infected tissue using rRNA probes has also been shown to be a sensitive method for detection and identification of *Candida*.[136] Finally, a repetitive DNA probe for *Pneumocystis carinii* has been shown to be more sensitive and specific than the standard immunofluorescence assay.[137] Despite their great promise, the usefulness of these DNA hybridization methods in clinical practice remains to be further integrated into clinical microbiology practices.

PCR for diagnosis of fungal infections

Apart from their use in epidemiology, PCR-based methods are especially attractive tools for diagnosing fungal infections. Many of the traditional diagnostic methods lack sensitivity in the clinical setting. PCR methods can potentially shorten diagnostic delay and improve patient outcomes. The high sensitivity of the PCR may however result in an unacceptably high number of false-positive assays. This section reviews the current literature on PCR-based methods for invasive aspergillosis, cryptococcosis, candidemia, candiduria and PCP.

PCR for invasive aspergillosis: Invasive pulmonary aspergillosis is a serious condition primarily affecting immunosuppressed patients. For example, attributable mortality for nosocomial *Aspergillus* pneumonia in bone marrow transplant recipients is 85%.[9] Although the recovery of *A. fumigatus* in BAL fluid may be highly informative in certain at-risk patients, cultures may be insensitive.[138,139] Several preliminary studies in anima have suggested that PCR on BAL fluid or serum might b a useful diagnostic method for invasive aspergillosis.[140,1 Some of the human studies on the use of PCR for diagno ing invasive aspergillosis are summarized in Table 2. potential problem with the PCR method for aspergillosis its extreme sensitivity, which results in positive tests sin ply due to contamination of fungal DNA from individua colonized but without evidence of disease.[142] Howeve studies using several different primers suggest that th method may be adequately specific, and therefore suitab to identify neutropenic patients at risk for invasive aspe gillosis.[141,143] One possible way to circumvent the proble of false positivity is to use competitive PCR, which the retically could elevate the detection limits of the assay ar only identify patients with a high burden of organism which might correlate with disease. Relatively few studi have used competitive PCR to diagnose fungal infection Bretagne and associates used this technology to investiga BAL samples from 55 neutropenic patients for the pre ence of *Aspergillus* DNA. Despite the sensitivity of th method (three out of three culture-proven invasive aspe gillosis), the high rate of false-positive tests (25%) cu rently indicates that further improvement in this assay ma be required before it can be of clinical value.[144] Obvious all molecular tests will need to be correlated with th clinical situation to improve their predictive value.

PCR for cryptococcosis: Oligonucleotide primers specif for *C. neoformans* have been designed so as to allow f specific identification of this organism in clinical sam ples.[145] Tanaka and colleagues compared traditional cultu to nested PCR for rDNA in patients with pulmonar cryptococcosis. The PCR method was positive in four five culture-positive samples, but negative results we obtained from all patients with histopathological confirmed but culture-negative cryptococcal lung di

ase.[146] The negative results from the latter group of patients raise questions as to the requirement for viable fungi in order for this particular primer combination to work. A larger clinical study comparing the sensitivity and specificity of PCR to the traditional methods of culture, India ink stains and cryptococcal antigen measurements seems warranted.

PCR for candidemia: According to the United States National Nosocomial Infection Study data, the prevalence of candidemia in hospitals is rising and the attributable mortality rate is 38%.[127] Rapid and sensitive diagnosis of candidemia and invasive candidiasis could improve the prognosis of these patients. A number of different PCR-based methods have been designed to detect *C. albicans* blood stream infections and have a reported sensitivity in the range of one to 100 organisms (colony-forming units) per ml of whole blood.[135, 147–155] Primer design enables two approaches for identification of pathogenic fungi. The first utilizes species-specific or genus-specific primers which hybridize to unique nucleic acid sequences in the genomic DNA. The second approach utilizes highly-conserved fungal elements such as ribosomal RNA genes as targets. An example of the former approach is utilization of mitochondrial-specific sequences for specific identification of *C. albicans*.[156] A slightly broader approach involves the use of primers which are specific for the chitin synthase gene in four medically important *Candida* species.[154] At the other extreme a highly conserved area in the 18S ribosomal RNA gene has enabled design of primers that are specific for a wide variety of medically important fungi.[135,149,157–159] PCR using these primers has been studied as a method for diagnosing a variety of fungal infections, both in neutropenic mice with candidemia as well as in selected clinical specimens. In the mouse model the detection threshold of the PCR method was 100 yeast cells, giving positive results in five of seven culture-negative animals and no false positives in uninfected controls.[149] An added benefit of the PCR methodology is the relatively short time needed for results, since a fungal pathogen can potentially be identified within eight hours.[157]

Van Deventer and colleagues designed a PCR assay using primers for the small subunit rRNA and compared the assay to the traditional method of blood cultures using neutropenic mice with invasive candidiasis.[150] Using a *Candida*-specific probe and Southern analysis, the PCR assay had the sensitivity of 100–150 organisms/ml of whole blood. In the animal model the PCR assay had a sensitivity of 100% when blood cultures were only sensitive in 67% of the infected animals. Moreover, animals colonized with *C. albicans* in the gastrointestinal tract did not have a positive signal on the PCR assay, which suggests high specificity. Other investigators have used primers specific for the enzyme cytochrome P-450 lanosterol–α-demethylase (L1A1) which is the target for azole antifungals.[153, 160] In one study the assay sensitivity in seeded clinical specimens such as blood or peritoneal fluid was close to 100 cells/ml. Compared to culture, the sensitivity and specificity for *C. albicans* were 71% and 95%, respectively.[153] Currently a prospective clinical trial is underway to better validate the usefulness of this method for bloodstream involvement.[160] Finally, the PCR technique has also been found to be useful for yeast species identification in cases of positive blood cultures, reducing the time required for identification from 3.5 days to seven hours.[161]

PCR for candiduria: Miyakawa and co-workers have shown that PCR for *C. albicans*-specific DNA fragment, EO3, was a sensitive (100 organisms/ml) and specific method for the detection of this organism in human urine.[156] Muncan and Wise compared PCR to a standard urine fungal culture in three groups of patients: patients with proven positive cultures; critically ill patients; and healthy volunteers. The sensitivity of the PCR assay was 100% in each group. Moreover, five of 60 critically ill patients had a positive PCR result on urine 24 to 48 hrs before a routine fungal culture became positive.[162] The authors proposed PCR testing of urine as an effective tool for early diagnosis of candiduria.

PCR for pneumocystosis: Based on phylogenetic analysis of the 16S ribosomal sequences of *Pneumocystis carinii*, this organism has been shown to be a member of the kingdom Fungi.[4] With the increased awareness for PCP in a growing immunosuppressed population, the need for rapid and sensitive tests for this fungus has prompted several groups to design diagnostic tests based on PCR for sputum, BAL samples or nasopharyngeal aspirates.[163–180] Some of the studies have reported both 100% sensitivity and specificity when compared to traditional methods. Recent data suggest that the sensitivity and specificity of a PCR test for PCP on respiratory samples from HIV-positive patients may be close to 100%.[172] In contrast, the sensitivity has been reported to drop to 25% and the specificity to 44% in patients with leukemia or lymphoma.[172] It has been shown that PCR may be 100-fold more sensitive than direct examination, which leads to lower specificity in a head-to-head comparison.[177] In animal models of PCP, semiquantitative PCR has been shown to correlate well with cyst counts in lung homogenates, suggesting usefulness of this method for following response to treatment.[181] A simple nested PCR assay was recently compared to immunofluorescence in a large, blinded, prospective study where 749 clinical specimens were analyzed by both methods.[182] The results suggested slightly higher sensitivity (94.8% versus 93.8%) and lower specificity (99.1% versus 100%) for the PCR method. The authors proposed that with the decreasing prevalence of PCP, and with the new antiretroviral treatments and thus the increase of negative samples for which longer time is required when the immunofluorescence detection method is used, the attractiveness of the PCR assay may actually increase.[182] On the other hand, PCR on serum for PCP is usually negative, except in patients with extrapulmonary pneumocystosis.[178,183]

PCR amplification and direct sequencing of *P. carinii* genes from sequential BAL samples has also been successfully used to distinguish relapses from *de novo* infections in HIV-infected patients.[125,184] Novel alternative methods to traditional PCR, such as *in situ* PCR or localized *in situ* PCR, have recently been proposed, allowing for site-specific amplification of the desired products.[185] The rRNA gene sequence of *P. carinii* has been amplified by using localized *in situ* PCR and detected by an immunohisto-

chemical detection system.[185] The benefit from using these novel methods for diagnosis of PCP in routine clinical practice remains to be evaluatcd.

CONCLUSIONS

The revolution in molecular biology has dramatically increased our ability to study fungal infections. Multiple tests are available for epidemiological purposes and these tests could be further improved. Several molecular methods are currently being developed for improved diagnosis of fungal infections and identification of fungal isolates. Molecular mycological methods have proven their value in advancing our understanding of clinical epidemiology. Most molecular methods are still only used in research or reference laboratories and need to be standardized and evaluated further before they can be routinely used in clinical microbiology laboratories. As yeast genome projects mature, it is likely that molecular chip technology, with its highly automated methods, will be applied for rapid identification and diagnosis of fungal infections and even for detection of antifungal drug resistance. The conversion from classical methods of culture and other conventional microbiological methods will likely be costly in the short term, but as technology improves, the speed and accuracy of these tests may actually become essential and also cost effective by reducing therapeutic imprecisions.

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Exhibit D

Therapy of *Candida* Infections: Susceptibility Testing, Resistance, and Therapeutic Options

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OBJECTIVE: Review the epidemiology of fungal infections, approved susceptibility testing methods, the scope of antifungal resistance, and advances in the treatment of fungal infections.

DATA SOURCES: MEDLINE databases (from 1966 to March 1998) were searched for literature pertaining to the epidemiology and management of fungal infections.

STUDY SELECTION AND DATA EXTRACTION: Articles were selected to assist in providing the reader an understanding of the epidemiology and management of fungal infections.

DATA SYNTHESIS: Fungi have emerged as an important class of pathogens. Even though fungi rank as the fourth most commonly encountered nosocomial bloodstream pathogen, and are associated with the highest mortality of commonly encountered pathogens, only within the past year have methods for conducting and guidelines for interpreting in vitro susceptibility tests been approved. Under the guidance of these standards, we have begun to understand important issues regarding fungi such as the scope and mechanisms of antifungal resistance. Although there has not been a significant addition to our antifungal armamentarium since 1992, advances in antifungal therapy have been realized with the reformulation of available agents and the delineation of the pharmacodynamic characteristics of several antifungals. Additionally, several new agents, including a new class of antifungals, probably will enter into clinical use within the next 5 years.

CONCLUSIONS: We have entered an era in which our understanding of fungi is increasing tremendously. Clinicians need to familiarize themselves with the current concepts surrounding the management of fungal infections in order to provide optimal care for their patients.

KEY WORDS: *Candida*, resistance, fluconazole, amphotericin B.

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AS RECENTLY AS 20 YEARS AGO, isolation of *Candida* from a clinical specimen was frequently labeled a culture contaminant and disregarded. In the span of 2 decades, however, we have witnessed the recognition and emergence of *Candida* spp. as a major cause of human disease.[1,2] From 1980 to 1989, rates of bloodstream infections caused by *Candida* spp. increased dramatically. Among the 124 National Nosocomial Infections Surveillance System (NNIS) hospitals, increases in the rate of candidemia over this 10-year period ranged from 75% among smaller nonteaching hospitals to 487% among large teaching hospitals.[1] Although many factors contribute to the increased incidence of fungal infections, and these issues are somewhat controversial, substantial morbidity and mortality have been linked to infections caused by *Candida* spp.[3] Even though the clinical significance of fungi is no longer in question, there remains a mystique surrounding the management of patients with fungal infections. Research into antifungal pharmacotherapy is currently one of the fastest growing areas in infectious diseases. As a result, there is a dire need to dispel antiquated concepts regarding antifungal therapy and expose clinicians to current patient management concepts. This article highlights recent developments that have served to improve our understanding for treatment of *Candida* infections and have the potential to impact our approach to patient care.

Emergence of Fungi as Human Pathogens

The 1980s gave witness to an explosive rise in the rate of fungal infections. According to NNIS system data,[2] during this decade, 30 477 fungal infections were reported among member institutions. This represents an increase in the all-site rate of fungal infections from 2.0 to 3.8 infections/1000 discharges ($p < 0.001$). The rate of fungemia increased from 1.0 to 4.9 cases/1000 discharges. Fungi currently rank as the fourth most commonly isolated pathogen among nosocomial bloodstream infections, accounting for approximately 8.0% of all hospital-acquired bloodstream infections.[4] Several factors have been linked to the rise in the occurrence of fungal infections. Included among these are the emergence of AIDS, exposure to broad-spectrum antimicrobials, increasing number of patients with neutropenia secondary to cancer therapies or transplant procedures, and expanded use of intravascular devices.[5-7] As medical technology advances, it is likely that patients will continue to evolve into a more severely ill population.

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Therefore, it is reasonable to assume that fungi will continue to play an increasingly prominent role as human pathogens.

Candida spp. account for more than 85% of all fungal bloodstream infections. Of the *Candida* spp. responsible for causing nosocomial bloodstream infection, the overall rank order of frequency of occurrence is *Candida albicans* (52%), *Candida glabrata* (20%), *Candida tropicalis* (11%), *Candida parapsilosis* (8%), and *Candida krusei* (5%).[4] Often debated is whether the percentage of infections caused by species other than *albicans* is increasing secondary to antifungal selective pressures. Several reports[8-12] support this trend; however, these data must be interpreted with an understanding of the tremendous amount of interinstitution variability associated with the frequency of isolation of various *Candida* spp. Reasons for this variability include the specific patient populations studied, institutional infection control practices, and differences in local antifungal usage patterns. The species variability among institutions was highlighted recently in a report[4] from the Surveillance and Control of Pathogens of Epidemiologic Importance program examining the frequency of isolation of species other than *albicans* from approximately 50 medical centers across the US. In this report, data regarding bloodstream isolation of fungi were evaluated collectively and according to geographic region (Table 1). Collectively among the regions, *C. albicans* accounted for 52% of all *Candida* isolates. However, when the incidence of *C. albicans* isolation was examined by region, the frequency of isolation ranged from a high of 70% in the Southwest to a low of 46% in the Northeast. Similar differences were also noted with respect to the frequency of isolation of other *Candida* spp. Despite these differences, the rank order of frequency of isolation among species remained relatively constant: *C. albicans* (most common) > *C. glabrata* > *C. tropicalis* > *C. parapsilosis* > *C. krusei*. These observations suggest that, although widespread surveillance data are important for tracking epidemiologic shifts among *Candida* spp., they do not provide reliable information regarding specific institutions and therefore should not be substituted for in-house surveillance programs.

Perhaps the only elements that are more impressive and frightening than the rapid emergence of fungi as recognized pathogens are the morbidity and mortality associated with infections secondary to these agents. One study[13] found *Candida* spp. to be the only organisms independently influencing mortality associated with nosocomial bloodstream infection (odds ratio for mortality = 1.84; p = 0.0035). In this evaluation of 1745 episodes of nosocomial bloodstream infections, *Candida* spp. were the fourth most frequently isolated pathogen, but were associated with the highest attributable 28-day mortality (35%). In contrast, attributable 28-day mortality for other commonly encountered nosocomial pathogens were 31% for *Pseudomonas aeruginosa*, 22% for *Staphylococcus aureus*, and 26% for *Escherichia coli*. Furthermore, Wey et al.[3] evaluated the attributable in-hospital mortality and excess length of stay associated with nosocomial candidemia among 88 case–control matched pairs. Crude mortality rates of 57% and 19% were noted for case and control patients, respectively, which resulted in a 38% attributable mortality (95% CI 26% to 49%). Analysis of 34 matched pairs of patients who survived revealed a length of hospitalization of a median of 70 and 40 days for case and control patients, respectively ($p < 0.0001$). These data serve to underscore the seriousness of fungal infections and drive home the urgency with which we should approach treatment of such infections.

In Vitro Testing of Antifungals

ANTIFUNGAL SUSCEPTIBILITY TESTING

As recently as 15 years ago, there did not appear to be a pressing need for in vitro antifungal susceptibility testing. Fungi were still vastly unrecognized as significant pathogens and treatment options for the management of systemic fungal infections were limited primarily to amphotericin B. As we began to understand the significance of fungi as pathogens and less toxic, yet effective, systemic antifungal agents became available for use in this setting, the advantages of being able to discriminate among therapeutic options became obvious. In 1997, 15 years after forming the Subcommittee for Antifungal Susceptibility Testing, the National Committee for Clinical Laboratory Standards (NCCLS) accepted and approved document M27 titled "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts."[14] Susceptibility tests performed according to these methods have demonstrated intra- and interlaboratory reproducibility comparable to results obtained for antibacterial testing.[15] According to these methods, one is then able to compare the relative potencies of various antifungal agents in vitro. Additionally, a rank order of species susceptibility is easily constructed. For example, upon examination of Table 2,[16] it is evident that the rank order of *Candida* spp. susceptibility to fluconazole and itraconazole is *C. albicans* (most susceptible) > *C. tropicalis* > *C. parapsilosis* > *C. glabrata* > *C. krusei*.

In order for in vitro susceptibility tests to be clinically useful, the in vitro data must correlate with in vivo results.

Table 1. Species Distribution of *Candida* Bloodstream Isolates: Nationwide and Regional[4]

	PERCENT BY REGION[a]				
SPECIES	SOUTHEAST (n = 133)	SOUTHWEST (n = 49)	NORTHEAST (n = 137)	NORTHWEST (n = 60)	ALL REGIONS (n = 379)
albicans	47	70	46	60	52
glabrata	23	10	23	13	20
tropicalis	10	8	17	7	11
parapsilosis	11	4	6	8	8
krusei	6	2	4	5	5
Other[b]	3	6	4	7	4

[a]Total number of hospitals was 37: Southeast 7, Southwest 8, Northeast 12, Northwest 10.

[b]Other *Candida* spp. include three *Candida lusitaniae*, one *Candida rugosa*, and two *Candida lipolytica*.

Correlating in vitro and in vivo findings proved to be a formidable task for several reasons. First, tremendous variation exists among dosing regimens that are employed with both azole antifungals and amphotericin B. Therefore, it becomes almost impossible to determine the effectiveness of susceptibility test results unless dosing regimens are explicitly stated. Second, the site of infection has to be considered, as this may affect drug penetration. Similarly, if an oral agent is used, drug absorption must be ensured. Third, host immune function and type of infection must be considered. It has long been realized that perhaps one of the greatest predictors of successful antifungal therapy is the return of the patient's immune function. In some cases this alone may be sufficient to result in a cure. Likewise, if the infection is secondary to a surgically correctable (e.g., abscess) or removable (e.g., catheter) source, a clinical cure may be realized despite infection with an extremely drug-resistant fungus. Taking all of these factors into account, the NCCLS subcommittee reviewed the available clinical data and established interpretative breakpoint values for fluconazole and itraconazole (Table 3).[17] These values were established with the intent that in vitro susceptibility does not always predict successful therapy, but rather, that in vitro resistance should often predict therapeutic failure.

Table 2. Susceptibility of *Candida* spp. to Fluconazole and Itraconazole[16]

SPECIES	MIC_{50} (μg/mL) FLUCONAZOLE	ITRACONAZOLE
albicans	0.25	0.06
tropicalis	0.5	0.12
parapsilosis	1.0	0.12
glabrata	8.0	0.5
krusei	32	1.0

MIC_{50} = minimum inhibitory concentration for 50% of the isolates tested.

Table 3. Proposed Interpretive Breakpoints for In Vitro Susceptibility Testing of *Candida* spp.[a,b]

ANTIFUNGAL	MIC (μg/mL)	INTERPRETATION	CLINICAL OUTCOME (% success)
Fluconazole	≤8	susceptible	97
	16–32	susceptible–dose dependent[c]	82
	≥64	resistant	60
Itraconazole	≤0.12	susceptible	90
	0.25–0.5	susceptible–dose dependent[c]	63
	≥1	resistant	53

MIC = minimum inhibitory concentration; NCCLS = National Committee for Clinical Laboratory Standards.

[a]Isolates of *C. krusei* should be considered resistant to fluconazole regardless of the reported MIC to fluconazole.

[b]Breakpoint values are applicable for MICs determined according to NCCLS-approved methods only.

[c]Isolates having an MIC in the susceptible–dose dependent range should be treated with fluconazole 400–800 mg or an appropriate dose of itraconazole with results in serum concentrations of ≥0.5 μg/mL.

ANTIFUNGAL TIME-KILL TESTING

Time-kill tests have long provided interesting and clinically useful data for antibacterials. Data gathered according to these methods have provided information regarding the relative rates of antibacterial activity, synergy/antagonism, and pharmacodynamic characteristics of numerous antibacterials. Methods governing antibacterial time-kill tests have been critically evaluated and standardized methods have been proposed by the NCCLS.[18]

Currently, time-kill data regarding the activity of antifungal agents are relatively scarce. The primary reason for the lack of antifungal time-kill data has been the controversy that has surrounded antifungal in vitro susceptibility testing; however, the acceptance of standardized susceptibility testing methods and demonstration of their utility has cleared the way for other methods of in vitro testing with the antifungals. Standardized guidelines concerning the implementation of antifungal time-kill test currently do not exist; however, a proposal for the creation of uniform testing methods is currently being studied.[19] Therefore, until standardized methods are developed, interpretation and comparison of data generated via different methods should be made with caution.

EVALUATION OF ANTIFUNGAL COMBINATIONS

The concurrent use of multiple antimicrobials can be justified under one of four circumstances: (1) the combination of agents results in synergistic activity; (2) the combination of agents slows or prevents the emergence of resistance; (3) the combination of agents results in broad coverage not attainable with a single agent; and/or (4) the combination of agents allows for the reduction of the dose of one or more of the agents, thus reducing toxicity. Before antimicrobial combinations are used clinically, the resultant effect of the combination must be evaluated in vitro to ensure that antagonism does not result.

Currently, one of two methods is typically used for the in vitro evaluation of antimicrobial combinations: the checkerboard method or time-kill techniques.[20] Two important differences exist between these two methods. First, checkerboard testing allows only for the determination of a single static end point, whereas time-kill methods provide a more detailed record of the drug–bacteria interaction over time. Time-kill methods therefore allow for the assessment and comparison of the rate and extent of the activity provided by the combination rather than only the extent as provided by checkerboard methods. The second difference stems from methodologic differences associated with the tests. According to the checkerboard method, the test isolate is exposed to both antimicrobials simultaneously. In contrast, drug exposure may be simultaneous, staggered, or sequential if time-kill methods are used. This fact is crucial if preexposure to one drug is necessary to observe antagonism against a second agent.

When discussions arise concerning the use of antifungal combination therapy, the fear that is most often voiced is that of azole-induced antagonism of the fungicidal activity of amphotericin B. This concern stems from the mechanisms of action proposed for these two classes of antifun-

gals. The primary mechanism of action cited for azole activity is inhibition of 14 α-demethylase-mediated conversion of lanosterol to ergosterol. Amphotericin B, on the other hand, is thought to bind to ergosterol embedded in the fungal cell wall. Theoretically, depletion of ergosterol binding sites by an azole should result in a decrease in the activity exhibited by amphotericin B.

In vitro time-kill studies have demonstrated that two conditions must be satisfied for azole-induced antagonism of amphotericin B to be expressed. First, fungi must be preexposed to the azole antifungal for approximately 8 hours prior to amphotericin B exposure.[21-23] Mechanistically, it is reasonable to assume that a certain amount of time must elapse in the presence of the azole for depletion of ergosterol to occur and hence antagonism to be expressed. Second, the action of the azole must persist in order to sustain expression of antagonism.[22] In vitro studies in which isolates of *C. albicans* that had been preexposed to fluconazole for 8 hours followed by subsequent removal of drug prior to addition of amphotericin B demonstrated rapid and full return of the fungicidal activity exerted by amphotericin B.[24] With these prerequisites for expression of antagonism in mind, it becomes apparent that both of these requirements can not be met using checkerboard testing methods. According to these methods, azole preexposure is not easily accomplished. Therefore, time-kill should be considered the method choice for the in vitro evaluation of antifungal combinations.

Antifungal Resistance

Resistance among fungal species to the currently available antifungal agents is an area of considerable interest. Until recently, it was extremely difficult to realize the true scope of resistance. Two factors that served to confound the antifungal resistance issue were lack of standardized susceptibility testing methods and little consensus regarding interpretation of susceptibility results. Although standardized susceptibility testing methods have now been approved and breakpoints have been proposed, interpretation of susceptibility test results still must be made with care. For example, according to the NCCLS proposed guidelines for in vitro susceptibility testing, results obtained with amphotericin B generally fall within a range of 0.25–1 μg/mL.[25] Since a majority of isolates exhibit a minimum inhibitory concentration (MIC) of 1 μg/mL or less for amphotericin B, isolates with an MIC greater than 1 μg/mL have been considered to be resistant. However, treatment failure occurred in 14 of 66 patients treated with amphotericin B from whom a *Candida* spp. with an MIC of 1 μg/mL or less was isolated.[25] This observation may either highlight the importance of host factors on the outcome of fungal infections or reveal the inability of current testing methods to adequately identify amphotericin B–resistant fungi.

Another example of the problems that can be encountered when attempting to evaluate the susceptibility literature is the variability associated with the interpretation of fluconazole results. Prior to the publication of the proposed breakpoint values, virtually no consensus existed regarding the interpretation of fluconazole resistance. Thus, values ranging from greater than 1.56 μg/mL to greater than 64 μg/mL have been used by investigators to define fluconazole resistance.[26,27] Although use of the proposed breakpoint values should help resolve some of the confusion surrounding the interpretation of fluconazole MICs, care must still be exercised when evaluating articles published prior to the introduction of the breakpoint values and when methods other than those approved by the NCCLS are employed for determination of MICs.

MECHANISMS OF RESISTANCE

Resistance among *Candida* spp. can be defined by two broad classifications: intrinsic and acquired. *C. krusei*, regardless of previous drug exposure, is a *Candida* spp. that is not inhibited by fluconazole; therefore, *C. krusei* is said to be intrinsically resistant to fluconazole. This is analogous to the intrinsic resistance exhibited by *Enterococcus faecalis* against cephalosporins. Since we know that *C. krusei* is resistant to fluconazole, we would never use this agent to treat an infection caused by this pathogen. Intrinsic resistance becomes problematic when selective pressures induced by antifungal use eradicate susceptible species and intrinsically resistant fungi such as *C. krusei* emerge as the dominant fungal flora. Several investigators[9-12,28] have documented the effects of antifungal selective pressures resulting in increased isolation of less-susceptible species such as *C. glabrata*, *C. krusei*, and *Candida lusitaniae*.

Acquired resistance is more troublesome than intrinsic resistance due to the unpredictability of its occurrence. This type of resistance can occur rapidly or emerge gradually. Although the mechanisms responsible for the expression of resistance may differ, the circumstances behind the emergence of resistance are common. Antifungal use results in selective pressures that select for the growth of the least-susceptible members of a fungal population. Whether the isolates exhibiting reduced susceptibility result from a mixed population of infecting organisms or emerge as the result of genetic mutation, the result is similar: a population of fungi resulting from the same species with higher MICs than the pretreatment isolates. Acquired resistance emerging secondary to each of these modes has been reported.[29-32]

Resistance occurring secondary to a genetic or structural alteration following drug exposure has been well documented among various bacterial species. There are now increasing numbers of reports describing similar genetic and structural alterations among fungal species as well. Table 4[33-46] summarizes mechanisms used by fungi to express resistance to amphotericin B and the azoles. Although resistance to amphotericin B is not considered to be common, resistance occurring secondary to both phenotypic[33] and genetic alterations resulting in the expression of resistance[34,35] have been reported. Several physiologic alterations are linked with azole resistance among fungi. Investigators[36,37] have reported upregulation and target site modification of 14 α-demethylase resulting in reduced susceptibility to fluconazole. Azole resistance has also been de-

scribed[38] secondary to the production and incorporation of alternate sterols such as cholesterol, zymosterol, fecosterol, campesterol, episterol, and sisosterol into the fungal plasma membrane. The most commonly cited and studied mechanism of azole resistance involves a reduction in the intracellular accumulation of these agents secondary to decreased drug permeability[39,40] and/or increased cellular drug elimination via efflux pumps encoded for by the *CDR1* and/or *MDR1* genes.[41-46] It is important to realize that cross-resistance among azoles is not complete[42] and that multiazole resistance appears to be more likely among isolates expressing the *CDR1* gene.[42,44]

Unlike bacteria, fungi do not appear to be capable of transferring genes' encoding for resistance. Furthermore, many *Candida* spp. lack a haploid growth state; therefore, a mutation coding for resistance is not likely to be expressed unless it is a dominant trait. Lastly, it appears that antifungal resistance, at least azole resistance, develops gradually and occurs as the sum of several genetic alterations.[47] Antifungal resistance will almost certainly become a more common clinical occurrence; however, it is not likely we will witness explosive changes in antifungal MICs across large segments of the fungal population as have been noted among bacteria.[48,49]

Therapeutic Strategies for the Treatment of Fungal Infections

Currently, our selection of agents for use in the management of systemic candidiasis is essentially limited to four agents: amphotericin B, fluconazole, itraconazole, and flucytosine. Excluding the various reformulated versions of these agents, there has not been a significant addition to our antifungal armamentarium since 1992. This is astounding if one considers that, on average, three to four new antibacterials are approved each year. Despite the absence of new antifungal entities as of late, there have been significant advances impacting our treatment approach for fungal infections. These advances include the reformulation of existing agents such as amphotericin B and itraconazole, characterization of the pharmacodynamic properties of antifungal agents, and investigation of new antifungal agents.

Table 4. Mechanisms of Antifungal Resistance

ANTIFUNGAL	MECHANISM OF RESISTANCE	REF.
Amphotericin B	alteration in β-1,3-glycan composition of fungal cell wall resulting in decreased drug permeability	33
	genetic alterations resulting in altered plasma membrane ergosterol content	34,35
Azole	reduced susceptibility of ergosterol synthesis to fluconazole resulting from reduced affinity for, or increased production of, 14 α-demethylase	36,37
	alteration in the sterol composition of the plasma membrane	38
	decreased permeability of the fungal membrane	39,40
	decreased intracellular drug accumulation secondary to the expression of drug efflux pumps	41–46

REFORMULATION

Despite being used in clinical practice for over 40 years, amphotericin B remains the most active antifungal agent we possess. Clinicians are often reluctant to initiate antifungal therapy with amphotericin B because of concerns regarding infusion-related events and nephrotoxicity. Interestingly, a retrospective study[50] describing amphotericin B toxicities over a 40-month period at the authors' institution found an extremely low incidence of amphotericin B–related adverse events. Renal toxicity was noted in only 15% of the patients receiving this agent. Although the patients included in this review appeared to tolerate amphotericin B extremely well, most of the patients were infected with *C. albicans* and were treated with a low average cumulative dosage of 162.5 mg. Furthermore, the authors pointed out that toxicity increased as the cumulative dosage of amphotericin B increased; therefore, caution should be exercised when extrapolating these data to other clinical situations. Since many patients require significantly higher cumulative dosages of amphotericin B, drug-related toxicity is still a pressing concern surrounding amphotericin B administration.

Recently, a tremendous amount of interest has been generated over the issue of complexing or encapsulating amphotericin B with lipids. There are currently three lipid formulations of amphotericin B available in the US: amphotericin B lipid complex (Abelcet, Liposome Co., Princeton, NJ), amphotericin B colloidal dispersion (Amphocil, Sequus Pharmaceuticals, Menlo Park, CA), and liposomal amphotericin B (AmBisome, NeXstar, San Dimas, CA). The subtle differences among these agents have been reviewed elsewhere.[51,52] Briefly, however, it appears that by encapsulating amphotericin B with a lipid layer, the toxicities associated with this agent can be minimized while the activity of the drug is maintained. The decreased renal toxicity observed with these agents appears to be the result of reduced renal accumulation of amphotericin B secondary to preferential accumulation within organs of the reticuloendothelial system (i.e., liver, spleen, lung).[53,54] The incidence of renal toxicity noted with the lipid formulations of amphotericin B varies among studies, with values ranging from 0% to 19%.[55-58]

Despite the approval of these three lipid-associated amphotericin B products by the Food and Drug Administration, there remain limited published data regarding the efficacy of these agents from well-controlled clinical trials. However, the existing published reports[55-57] demonstrate that the lipid formulations are at least as effective as amphotericin B deoxycholate.

Another product that has benefited from reformulation is itraconazole. Although itraconazole demonstrates good in vitro activity, its use has been limited secondary to poor oral bioavailability and lack of a parenteral product. Recently, however, an oral solution of itraconazole formulated in hydroxypropyl-β-cyclodextrin has been approved. When administered as the capsule formulation, itracona-

zole 200 mg (fed state) results in an AUC_{0-24} of approximately 2682 ng/mL • h.[59] In contrast, a similar dose of itraconazole administered as the oral solution results in an AUC_{0-24} of approximately 4505 ng/mL • h. This translates into a 70% increase in the oral bioavailability of itraconazole secondary to its reformulation. Furthermore, an intravenous formulation of itraconazole is also being developed which should further increase the clinical utility of this agent.

PHARMACODYNAMICS

Characterization of the pharmacodynamic properties of antibacterial agents has greatly improved our understanding of how to optimize the activity and therapeutic efficacy of these compounds. Despite the impact that such data have had on the clinical use of these drugs, similar data regarding antifungals have been relatively scarce. More pharmacodynamic data for antifungals, however, are beginning to emerge. Employing time-kill methods, Klepser et al.[60,61] described the relationships between concentrations and antifungal activity of amphotericin B and fluconazole against *C. albicans* and *Cryptococcus neoformans*. In these studies, amphotericin B was found to exert rapid fungicidal activity which increased in rate and magnitude when test concentrations of the antifungal were increased. Similar findings were reported[62] against isolates of *C. albicans*, *C. tropicalis*, and *C. glabrata*. The concentration-dependent activity reported for amphotericin B by these investigators is reminiscent to that which has been described with the aminoglycosides.[63]

Although clinical trials have not been designed specifically to examine the pharmacodynamics of amphotericin B, in vivo data supporting these findings do exist. For example, investigators[64-68] have evaluated several amphotericin B dosing regimens for the treatment of cryptococcal meningitis. In general, lower mortality was reported among the studies when higher doses of amphotericin B were used. Mortality decreased from 14% (9/63 patients) with a dose of 0.3 mg/kg/d or greater of amphotericin B (no flucytosine)[64] to 5.5% with a dose of 0.7 mg/kg/d (10/179 patients with flucytosine and 11/202 patients without flucytosine)[65] and 3% (1/31 patients) with an amphotericin B dose of 1 mg/kg/d plus flucytosine.[67] Improved outcomes among patients treated with higher doses of amphotericin B may be linked to more rapid fungal eradication resulting from increased drug concentrations at the site of infection.

In contrast, relatively concentration-independent fungistatic activity has been reported for fluconazole and other azole antifungals.[23,60-62] For these agents, maximal fungistatic activity is generally observed at concentrations equal to one to two times the MIC of the test organisms.[60,61] As with amphotericin B, no well-controlled clinical trials exist specifically designed to evaluate the dose–response relationship with the azoles. However, in vivo data do exist that substantiate the concentration-independent relationships that have been observed in vitro.[69] Therefore, according to these data, fluconazole dosing regimens should be employed that result in concentrations at the site of infection slightly higher than the MIC of the pathogen. Interestingly, this goal appears to be satisfied if one follows the dosing recommendations[17] that accompany the breakpoint values published for fluconazole and itraconazole.

Data regarding the postantifungal effect (PAFE) of clinically used agents are also remarkably scarce. However, a few reports[70,71] exist that suggest an extended PAFE for amphotericin B (0.5 to >10 h) and flucytosine (0.8–7.4 h) against *C. albicans* and *C. neoformans*. In contrast, minimal to no PAFE has been reported with fluconazole and the other azoles.

INVESTIGATIONAL ANTIFUNGAL AGENTS

With the limited options available for the treatment of severe mycosis, there is an unquestionable need for the development of new antifungal agents. This is especially evident for the treatment of invasive aspergillosis, histoplasmosis, blastomycosis, penicillosis, and resistant candidiasis. Currently, several new triazole antifungals are being evaluated in clinical trials for treatment of systemic and oral candidiasis and invasive aspergillosis.

Voriconazole is a new broad-spectrum triazole antifungal agent that entered Phase III clinical trials in 1996. In addition to possessing excellent activity against *Candida* spp. and *C. neoformans*, voriconazole appears to have fungicidal activity against *Aspergillus* spp.[72,73] Similar to itraconazole, voriconazole is a highly lipophilic compound metabolized by cytochrome P450 enzymes and demonstrates a nonlinear pharmacokinetic profile. Voriconazole may become preferred over itraconazole for treatment of *Candida* and *Aspergillus* infections because of improved drug bioavailability noted with the oral formulation and the availability of an intravenous voriconazole preparation.[74] Preliminary results[75,76] from clinical trials investigating voriconazole therapy in invasive aspergillosis refractory to therapy with amphotericin B, itraconazole, or both have reported enhanced efficacy (~70% response) and tolerability with both intravenous and oral voriconazole therapies.

Another extended-spectrum triazole under development that has exhibited enhanced antifungal activity over currently available antifungals is SCH 56592. In comparison with other azoles, SCH 56592 has been shown[77] to be comparable to itraconazole and more active than fluconazole against virtually all pathogenic *Candida* spp. tested. Although SCH 56592 has been reported[78] to be effective against fluconazole-resistant strains of *C. albicans* and *C. tropicalis*, Pfaller et al.[77] noted that the MICs for SCH 56592 were elevated among fluconazole-resistant strains, thus suggesting cross-resistance.

BMS-207147 is another investigational triazole antifungal agent with an expanded spectrum of activity. Similar to voriconazole, BMS-207147 has expanded coverage against *Candida* spp. compared with fluconazole and is also active against *C. neoformans*, *Aspergillus* spp., and various other filamentous fungi.[79]

Perhaps the most interesting class of antifungals currently being evaluated in clinical trials are the echinocandins. Echinocandins act by inhibiting glucan synthesis via inhibition of the enzyme 1,3,-β-D-glucan synthase.[80] Because

glucan synthesis is a vital process for maintaining fungal cell wall integrity, these drugs may exhibit fungicidal activity against a broad range of fungal pathogens including *Candida* and *Aspergillus* spp. A notable omission in the spectrum of activity of these agents is *C. neoformans*.[80,81] With a distinct mechanism of action that does not involve ergosterol, the echinocandins have the potential for use in combination regimens with currently available antifungal agents. Two agents in this class that have demonstrated promising in vitro activity are LY303366 (Lilly) and MK-0991 (Merck).

MK-0991 is currently undergoing clinical investigation. Preliminary results[82] from Phase II trials comparing intravenous 2-week therapy with MK-0991 50 or 70 mg/d and amphotericin B 0.5 mg/kg/d for endoscopically documented *Candida* esophagitis have been reported. Clinical response (reduction in symptoms and endoscopic lesions) was documented in 85.1% of the MK-0991 versus 66.7% in the amphotericin B group. Adverse effects during treatment with the MK-0991 were uncommon. Additional human clinical trials and pharmacodynamic studies will further help to define the role of these novel antifungals.

Summary

A tremendous amount of progress has been made recently with regard to improving our understanding of the pathogenesis and treatment of fungal infections. Despite these advances, however, our knowledge concerning these organisms is still in its infancy. The current surge in interest in developing novel antifungal agents and understanding antifungal resistance has positioned this area to grow exponentially over the next decade. It is now more important than ever that clinicians understand the role fungi play in human disease and remain current with respect to therapeutic strategies.

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EXTRACTO

OBJETIVO: Revisar la epidemiología de las infecciones fúngicas, los métodos aceptados para la valoración de la susceptibilidad, el alcance de la resistencia antifúngica, y los avances en el tratamiento de las infecciones por hongos.

FUENTES DE INFORMACIÓN: Se revisó la base de datos MEDLINE (1966–marzo 1998) para los trabajos relacionados con la epidemiología y tratamiento de las infecciones fúngicas.

FUENTES Y MÉTODO DE EXTRACCIÓN DE LA INFORMACIÓN: Se seleccionaron artículos para facilitar al lector el conocimiento de la epidemiología y el tratamiento de las infecciones fúngicas.

SÍNTESIS: Los hongos han aparecido como un importante grupo de patógenos. Aunque los hongos se sitúan como el cuarto grupo más frecuente de patógenos nosocomiales en el torrente circulatorio y se han asociado con la mayor tasa de mortalidad de los patógenos más frecuentes, solo durante los últimos años se han aprobado métodos y directivas para la realización e interpretación de los estudios de susceptibilidad in vitro. Bajo las directrices de estos procedimientos hemos empezado a entender hechos importantes respecto a los hongos, como el alcance y mecanismos de la resistencia antifúngica. Aunque desde 1992 no ha habido aportaciones importantes al arsenal terapéutico antifúngico, se han llevado a cabo avances en el tratamiento antifúngico con la reformulación de agentes terapéuticos ya disponibles y se han perfilado las características farmacodinámicas de distintos antifúngicos. Además, está previsto que varios nuevos productos, incluyendo una nueva clase de antifúngicos, entren en ensayos clínicos dentro de los próximos 5 años.

CONCLUSIONES: Hemos entrado en una era en la que nuestro conocimiento de los hongos está aumentando notablemente. Los clínicos necesitan familiarizarse con los conceptos modernos respecto al tratamiento de las infecciones por hongos a fin de aplicar el tratamiento más adecuado para sus pacientes.

JUAN ROCA-ACÍN

RÉSUMÉ

OBJECTIF: Revoir l'épidémiologie des infections fungiques, les méthodes approuvées pour tester la sensibilité aux antifungiques, la portée de la résistance à ces médicaments, et les percées dans le traitement de ces infections.

REVUE DE LITTÉRATURE: L'information provient d'une recherche dans la banque informatisée MEDLINE des articles publiés sur l'épidémiologie et le traitement des infections fungiques depuis 1966 jusqu'en mars 1998.

SÉLECTION DES ÉTUDES ET DE L'INFORMATION: Les articles retenus sont ceux qui permettent de mieux comprendre l'épidémiologie et le traitement des infections fungiques.

RÉSUMÉ: Les fungi sont devenus une classe importante de pathogènes. Même si les fungi sont la quatrième source la plus fréquemment rencontrée de pathogènes responsables d'infections nosocomiales et sont associés au plus haut taux de mortalité, ce n'est que lors de la dernière année que des méthodes pour effectuer des tests de sensibilité in vitro et des lignes directrices pour les interpréter ont été approuvées. Ces standards ont permis de mieux comprendre des sujets fondamentaux comme les mécanismes de résistance et leur portée thérapeutique. Bien qu'il n'y a pas eu d'ajouts significatifs de nouvelles molécules à la batterie de médicaments antifungiques existants, une percée a été effectuée par la reformulation des agents disponibles, et par la précision des caractéristiques pharmacocinétiques de plusieurs d'entre eux. De plus, plusieurs nouveaux agents, dont une nouvelle classe d'antifungiques, doivent faire leur entrée pour utilisation clinique d'ici les 5 prochaines années.

CONCLUSIONS: Nous sommes dans une période où notre compréhension des fungi augmente considérablement. Les médecins cliniciens ont besoin de se familiariser avec les connaissances actuelles concernant le traitement des infections fungiques de façon à assurer leurs patients des soins optimaux.

DENYSE DEMERS

Candida albicans: An opportunistic threat to critically ill low birth weight infants

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Major advances in the management of critically ill low birth weight (LBW) infants have increased their survival. Yet the clinical course of these infants is complicated by the emergence of opportunistic microbial pathogens. Most importantly, serious infections from opportunistic fungi, such as Candida albicans, *have produced systemic disease in vulnerable LBW infants. Invasive* C. albicans *infection is generally difficult to manage and is associated with high morbidity and mortality. Because the infection has an insidious and rapid course, the critical care nurse and advanced practice nurse need to provide key prevention and early treatment measures.*

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Within the last 20 years major strides in perinatal medicine have enhanced the survival of critically ill low birth weight (LBW) infants. It is ironic that these same advances in neonatal critical care have contributed to the emergence of opportunistic microbial pathogens that seriously threaten the survival of LBW infants. Opportunistic microorganisms flourish in vulnerable infants, yet they pose no threat to those with a healthy and intact immune system. These pathogens contribute to excessive medical and economic burden well beyond that of the infant's underlying disease and as such present a formidable challenge to the advanced practice neonatal critical care nurse.

Candida albicans is one such microorganism that has evolved from a commensal organism into a major opportunistic pathogen that strikes susceptible hosts, such as the LBW infant.[1,2,3] Unfortunately, it too frequently interrupts and complicates the critical care course of LBW infants. Because the infection is difficult to diagnose and treat, it is associated with high morbidity and mortality, particularly in the smallest and most seriously ill infants.[3,5,6] Knowledge of factors that modify critical host-microbial interactions form the foundation for critical care nursing actions designed to prevent opportunistic fungal infections and effectively treat infants beset by them.

This article describes the advanced pathophysiology of fungal infections and nursing actions designed to reduce the incidence and severity of these infections. This material can be used by neonatal practitioners and nurse educators to educate and update nursing staff and students on prevention and management strategies for opportunistic fungal infections. Advanced practice nurses can also use the information to develop and refine unit policy concerning infection-control practices and to implement strategies aimed at reducing infant risk for fungal infection.

PATHOPHYSIOLOGY

The increase in invasive fungal infections over the past decade is striking.[2] Overall, in hospitalized patients, the rate of candidal bloodstream infections increased as much as 487% during the 1980s.[4] *Candida* was reported to be the fifth most common isolate from blood cultures in the United States from 1986 to 1990.[7] In the neonatal intensive care unit (NICU), the impact of invasive *Candida* infections is considerable. Before the 1970s, systemic candidiasis in neonates was rare. Now *Candida* is a major cause of nosocomial infections in LBW infants and causes significant mortality.[1,8,9,10] *Candida* infections are common in the nursery as oral thrush or diaper dermatitis, but it is the invasive form of candidiasis that has been described as a "nursery epidemic" unique to the 1980s[3] and that continues to be a significant threat to the survival of LBW infants in the 1990s.[3,11,12] Overall, the incidence of *Candida* infections ranges from 1.6% to 5.9% in very low

birth weight (VLBW) infants (< 1,000 grams); in the extremely LBW infant (< 800 grams) the incidence of disseminated candidal infections can be as high as 18% to 20%.[5] Of the *Candida* species, *C. albicans* is most commonly associated with disease.[3,9,10]

Colonization

Typically, infants become colonized with *Candida* soon after birth. *C. albicans* can be isolated from the skin and oropharynx of most infants, especially LBW infants, in the early postnatal period.[5] The occurrence of colonization for various sites in LBW infants is listed below:

- Rectal, 83%
- Oropharyngeal, 46%
- Groin, 42%
- Urinary tract, 33%
- Endotracheal, 30%

Colonization from the maternal vaginal tract during birth is a prime mode of *Candida* transmission. This is especially true in pregnant women with vaginal candidiasis, the incidence of which approximately doubles during pregnancy. At birth, infants swallow *Candida*, and if the infant has other significant risk factors, fungal overgrowth proceeds rapidly.[6] Any intestinal insult (for example, ischemia, presence of necrotizing enterocolitis) can further facilitate the translocation of *Candida* across the intestinal wall or allow direct access to the vascular system.[7,16,17] Interruption of the initial colonization of the infant at birth offers promise for decreasing the incidence of systemic *Candida* infections in high-risk infants.

Risk factors and neonatal *Candida* infection

Disseminated *Candida* infections are generally associated with so-called late, late onset sepsis in the infant, in that it is a major pathogen of those critically ill infants with extended stays in the NICU.[1,11] Yet recently, the emergence of invasive fungal dermatitis has been reported in VLBW infants at a mean postnatal age of 9 days.[8] Colonization with *C. albicans* does not always produce invasive disease. Only in susceptible hosts such as the LBW infant—where both inadequate immune defense mechanisms and risk factors associated with clinical management are present—can *Candida* become invasive.

The primary risk factor for invasive neonatal candidiasis is low birth weight.[1,3] The LBW infant is deficient in both innate (nonspecific) and adaptive immunity. Other major risk factors that compound the susceptibility of the infant to *Candida* infection are listed below.[1,3,10]

- Prolonged antibiotic use
- Prolonged use of intravascular catheters
- Hyperalimentation
- Gastrointestinal surgery/disease
- Skin breakdown
- Malnutrition
- Prolonged intubation
- Intralipid infusions
- Glucocorticoids.

The immature skin of LBW infants lacks the normal barrier function that prevents penetration of commensal organisms such as *Candida*. Excessive moisture and warmth of the skin facilitate *Candida* growth and any break in skin integrity by trauma or abrasions allow *Candida* penetration. Other factors that compound the risk of candidiasis include long hospitalizations, proliferative use of broad-spectrum antibiotics that facilitate *Candida* overgrowth, prolonged hyperalimentation, fat-emulsion infusions (intralipids), hyperglycemia, and prolonged intubation.[3,15,18]

Candida is known to adhere to the plastic surfaces of catheters and tubing commonly used in critical care. Once adherence occurs, colonization occurs rapidly. Although these risk factors are associated with candidiasis, the strength of these associations to invasive *Candida* infection is limited by the retrospective nature of the studies, small sample size, and limited use of multivariate analysis to define independent risk factors.[16] In summary, it is the synergy between risk factors and the immaturity of host defense of the LBW infant that disrupts the normal host-commensal relationship and leads to invasion and dissemination of *C. albicans*.

Glucocorticoids and neonatal *Candida* infection

Glucocorticoids, which are widely used prenatally and postnatally, are also a potential risk factor for the development of invasive fungal infections. However, the risk of infection, in general, following antenatal glucocorticoid therapy, is controversial.[19-21] Glucocorticoids, at concentrations reported in umbilical cord blood, inhibit neonatal (but not adult) polymorphonuclear neutrophil leukocyte (PMN) cell chemotaxis *in vitro*.[19] Infants treated postnatally with glucocorticoids are reported to have an increased colonization with *Candida*.[20] However, it is not known whether this is due to glucocorticoid-induced immunosuppression or whether the glucocorticoid administration merely indicates infants in a more unstable condition with a higher risk for colonization with *Candida*. A recent hospital case-control study found that infants with disseminated *Candida* infections were 7.5 times as likely to have received intravenous hydrocortisone (I.V.) than infants who did not have *Candida*.[21] Another recent study found that the postnatal use of glucocorticoids was associated

with invasive fungal dermatitis in VLBW infants.[8] Glucocorticoids have important therapeutic indications in the LBW infant, but they may play a role in infectious risk as well.

In addition to therapeutic purposes, endogenous levels of glucocorticoids from adrenocortical secretion also increase in response to activation of the hypothalamic-pituitary-adrenocortical axis. Environmental stressors are potent stimuli of this axis, and typically the LBW infant in the NICU environment is besieged with stressors, both physical and psychological. Compelling evidence indicates that neutrophils from "stressed" infants have an even greater impairment in immune function.[23] Our laboratory has recently found that infants with more physiologic stress, as measured by the score for Neonatal Acute Physiology, have a greater impairment in antifungal defense (presented at the Pediatric Research Society meeting, 1997.) This suggests that a stressed infant is less immunocompetent and more vulnerable to opportunistic infection. Although intriguing, the relationship between stress and immune function in the infant is primarily based on a small number of critically ill infants and needs further study.

Pathogenesis of candidal infections

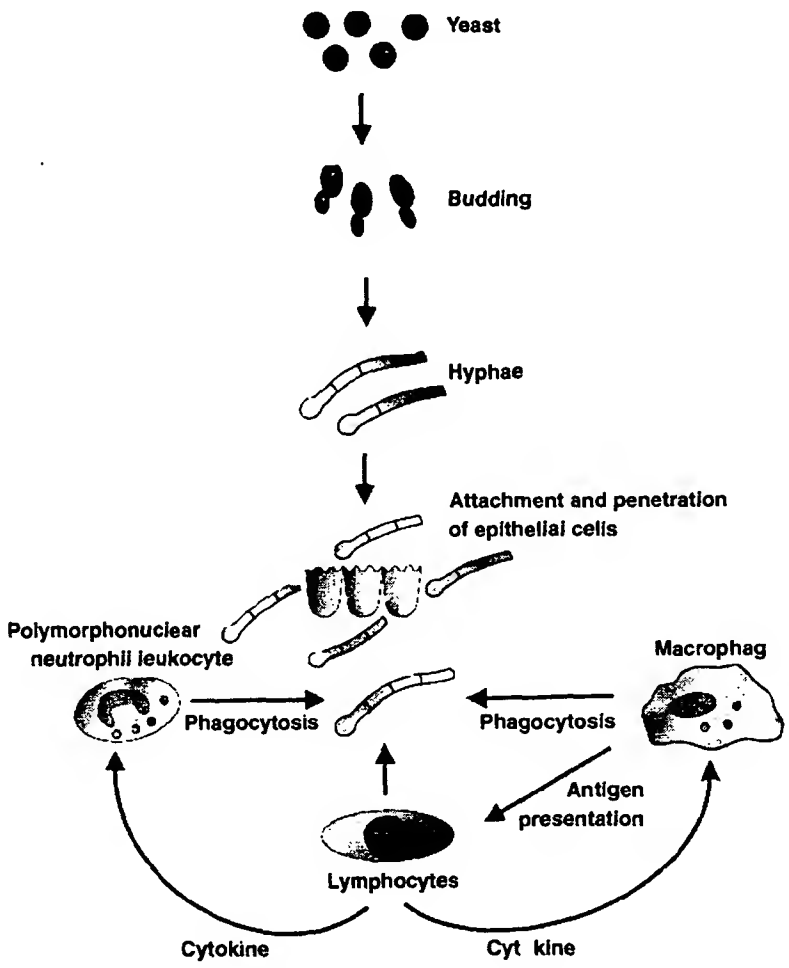
Candida albicans is Latin for "dazzling white," which describes the appearance of colonies of *Candida*. It is a dimorphic fungus, meaning it has the ability to transform itself into two different forms. *Candida* exists mainly as single-celled oval yeast cells, which under appropriate conditions bud asexually, forming chains of elongated budding cells (pseudohyphae) that transform into true hyphae (Figure 1).

Pseudohyphae and true hyphae are seen in tissues during infection. The hyphal form of *Candida* is associated with increased virulence and tissue invasion, in part because yeast hyphae are more resistant to phagocytic killing. Although environmental and host factors are suspected, researchers don't yet understand what triggers the conversion of *Candida* from the yeast to the hyphal form. *Candida* does not secrete any toxins,

FIGURE 1

Pathogenesis of candidal infection

Candida albicans exists as single-celled yeasts that in a susceptible host undergo budding and transformation to hyphae. The hyphal form is more virulent and attaches to epithelial cells of the host. The immune response to *Candida* invasion includes phagocytosis of *Candida* by macrophages and polymorphonuclear leukocytes and direct effects by lymphocytes. Macrophages present fungal antigen to lymphocytes, which further amplify the immune response through the secretion of cytokines. This immune response is deficient in the LBW infant.



but in susceptible hosts, the hyphae attach to epithelial cell membranes and penetrate host cells. The epithelial surface of the skin and mucus membranes are prime targets. The severity of *Candidal* infection is dependent on size of the inoculum, the virulence of the organism and, most importantly, the strength of the host defense system.[24]

Host defense against *Candida*

Host defense against *Candida* involves innate and adaptive immune responses. Innate immunity is the first line of defense and involves nonspecific cellular and humoral factors that prevent overt infection. Although serum factors such as opsonins and complement may decrease the survival of *Candida*, the main form of innate defense is phagocytosis and intracellular killing of engulfed fungi by PMNs and macrophages.[24]

Once *Candida* becomes invasive, cell-mediated immunity (CMI) is activated. CMI is initiated by macrophages that not only directly eliminate fungi but also present fungal antigen to T lymphocytes. The T lymphocytes secrete various cytokines that markedly augment the immune response.[25]

Additionally, large granular lymphocytes directly interact with and eliminate fungi and also produce cytokines (Figure 1). Cytokines that are important to host antifungal defense include interleukin-2, tumor necrosis factor, interferon (IFN), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cytokines are crucial to recruiting and activating immune cells in the area of tissue invasion.[25,26]

Immunologic basis for enhanced susceptibility to *Candida albicans*

The immune system of the LBW infant is characterized by multiple immunologic deficiencies that increase the infant's risk of opportunistic fungal infections.[27] These include both inadequate innate host defense mechanisms (for example, phagocytic cell function), as well as inadequate adaptive host defense mechanisms (for example, CMI).[27] Functional defects in neonatal PMNs and macrophages include defects in adherence to invading microorganisms, which is linked to impaired induction of adhesion molecules (Mac-1; γ_M/β_2) by chemotactic stimuli.[28] Adhesion molecules are required for binding immune cells to *Candida*, the first step in antifungal action.[29]

Low birth weight infants have numerous immune deficiencies that allow fungal infection to become invasive.

Neonatal immune cells also have marked impairments in deformability, phagocytosis, and intracellular killing.[30] Recently, investigations using neonatal animal models indicate that lung macrophages have reduced anticandidal activity and may increase the risk of respiratory fungal infections in the infant.[31] Data from human studies suggest that functional defects in PMNs are even more pronounced in the critically ill or stressed LBW infant.[23] As a result of these immune defects, LBW infants have an impaired ability to deliver phagocytes to a site of fungal invasion, and this allows the infection to become invasive and produce disseminated infection.

Compounding the deficiencies in innate immunity, CMI in the LBW infant is characterized by impaired T-cell production of cytokines. This further adds to the impaired phagocytic cell function observed in the neonate.[32] (Interesting preliminary results suggest that lymphocytes from male preterm infants have significantly less antifungal activity than lymphocytes from female infants.[33]) Because both phagocytic cell function and CMI are essential for defense against opportunistic fungal infections, the LBW infant has a profoundly reduced capacity to resist, localize, and eradicate *C. albicans*.

As indicated above, cytokines are immunoregulatory factors produced by immune cells that are needed to orchestrate an immune response. Without cytokine activation, immune effector cells have limited antifungal activity. Neonatal T lymphocytes (both $CD4^+$ and $CD8^+$) are deficient in their ability to produce IFN-γ, which is an important activator of neutrophils and macrophages and augments killing of *Candida*.[34] Neonatal T lymphocytes produce 10-fold less IFN-γ than adult T lymphocytes under similar conditions.[32] Neonatal monocytes obtained from umbilical cord blood of healthy full-term infants have a decreased ability to produce colony stimulating factors (CSFs), such as GM-CSF, when compared to adult monocytes. However, neonatal (full-term) PMNs have adultlike affinity and numbers of receptors for GM-CSF,[35,36] suggesting that it may be possible to therapeutically upregulate their activity against *Candida* by exogenous administration of CSFs. Although not used for all premature infants, G-CSF is currently used in neutropenic infants at risk for sepsis and has been shown to significantly increase the absolute neutrophil count.[36] How effec-

tive G-CSF may be in preventing fungal infections has yet to be determined.

Defects in cytokine production undoubtedly contribute to the susceptibility of the LBW infant to fungal invasion; however, of equal if not greater importance is the impaired ability of immune effector cells to respond to cytokine activation (that is, neonatal immune effector cells may not be optimally activated by cytokines). Marodi et al.[37] investigated IFN-induced candidacidal mechanisms using macrophages from umbilical cord blood obtained from healthy full-term neonates. Those data suggest that although full-term neonatal macrophages have an adult-like capacity to ingest and kill both opsonized and unopsonized *Candida*, activation of neonatal macrophages by IFN-γ was significantly less than that of adult macrophages.

Treatment

Infants who have systemic candidiasis should be treated by removing all factors that predispose them to systemic candidiasis. This includes indwelling catheters and broad-spectrum antibiotics. Systemic antifungal therapy should begin as early as possible. The infant should be examined for other foci of disease. Pharmacologic management of disseminated candidiasis in neonates is severely limited because of the propensity of most antifungal agents for toxic effects, particularly in infants with compromised renal function (Table 1). While there are various antifungal agents available for use in the treatment of systemic fungal infections, amphotericin B remains the drug of choice for treatment of neonates. It may be used alone or in combination with flucytosine (5-fluo-

TABLE 1

Antifungal agents for treatment of neonatal candidiasis

Antifungal agent	Dose	Adverse effects	Nursing actions
Amphotericin B	• 0.25-1 mg/kg/day over 4-6 hr[13,38] • Usual duration: 3-4 wks[13,38] (Duration of therapy depends on the site and severity of infection.)	• Infusion-related reactions—fever, tachycardia, cardiac arrhythmias (rare), thrombophlebitis[38,42,43] • Nephrotoxicity—oliguria, elevated blood urea nitrogen (BUN) and creatinine levels, renal tubular acidosis[38,42,43] • Electrolyte disturbances—hypokalemia (more common), hyperkalemia[38,42,43] • Miscellaneous—bone marrow depression, anemia, thrombocytopenia, tremors[38,42,43]	• Record fluid intake and output. • Check urine specific gravity. • Monitor BUN, creatinine, sodium and potassium levels. • Reverse sodium depletion and keep patient well hydrated to prevent nephrotoxicity. • During drug administration, monitor vitals frequently. • Assess for fever, tachycardia, cardiac arrhythmias, hypotension. • Observe intravenous site. • Monitor blood counts.
Flucytosine	• Newborns to 1 month of age: 50-100 mg/kg/day; given once daily[42,43] • Infants over 1 month: 100-150 mg/kg/day; P.O. every 6 hrs[42,43] • Dosage must be adjusted in presence of renal insufficiency[42,43] • Usual duration: 3-4 weeks[42,43] (Duration of therapy depends on the site and severity of infection.)	• Bone marrow depression: (Observed after 10-26 days of therapy)[13,42,43] —anemia —neutropenia —thrombocytopenia • Gastrointestinal disturbances—diarrhea, colitis, especially when used in combination with amphotericin B[13,42,43] • Hepatitis (elevated liver enzyme levels)[13,42,43] • Elevated BUN and creatinine levels[13,42,43]	• Monitor blood counts, BUN, and creatinine, and liver enzymes levels. • Assess for gastrointestinal disturbance: abdominal distention, elevated gastric residuals, diarrhea, feeding intolerance.

rocytosine or 5-FC) if it has been determined that the culpable strain of *Candida* is sensitive. The azole antifungal drugs such as ketoconazole, fluconazole, and itraconazole are oral agents and, because many premature infants are unable to tolerate oral medications, their use is limited in the NICU.[13,38] Specific nursing actions related to amphotericin B and flucytosine are addressed below.

NURSING IMPLICATIONS

The advanced practice critical care nurse plays a pivotal role in providing leadership for the NICU staff in preventing, identifying, and effectively managing the infant with candidiasis. Advanced knowledge of the dynamics of critical host, microbial, and environmental factors that interact and lead to invasive disease will prepare the nurse to prevent and control the potential deleterious effects of fungal infections.

Decrease risk factors

To prevent the development of *C. albicans* infection, the advanced practice nurse must be vigilantly aware of the presence of risk factors for *Candida* infection. At the time of an infant's NICU admission, nurses should identify infant risk factors related to maternal-fetal perinatal history. Because the presence of maternal infection significantly increases the infant's risk of significant *Candida* colonization at birth, nurses must evaluate any existing maternal vaginal candidiasis and/or a history of maternal chronic recurring vaginal candidiasis. Other indicators of maternal infection include maternal fever, foul-smelling amniotic fluid and/or vaginal discharge, and elevated maternal leukocyte count. Prematurity and/or low birth weight is the primary risk factor for contracting opportunistic fungal infections such as *C. albicans*. Increased severity of illness requires the use of more invasive care measures, and the presence of intravascular lines, prolonged intubation, hyperalimentation, and intralipid infusions alerts the nurse to the synergistic effect of these risk factors with the infant's limited immune defense mechanisms.

The old adage that the skin is the first line of defense is especially pertinent to the LBW infant. The barrier function of the LBW infant's immature skin is severely limited and fungal dermatitis is a significant problem in the NICU, because fungal dermatitis in an immature host can readily progress to invasive disease.[8] The prolific use of invasive care measures further compromises the LBW infant's skin integrity and compounds the infant's risk of *Candida* colonization and invasion. The advanced practice nurse should develop and maintain high standards for infant skin care in the NICU.

Antibiotic ointments at catheter insertion sites may enhance fungal colonization.

Although intravascular catheters serve as the portal of entry for invasive fungi, antibiotic ointments at the catheter site are thought to play a role in fungal invasion by inhibiting bacterial flora and enhancing fungal colonization.[38] Nursing staff can be instructed about the rationale for avoiding excessive use of antibiotic ointments.

Normal colonization with *C. albicans* occurs in the gastrointestinal (GI) tract. This is significant for two reasons. First, candidal colonization of the GI tract is markedly increased when an infant is on prolonged antibiotic therapy because of lack of competing commensal organisms. Candidal overgrowth together with the impairment of the mucosal barrier function of the GI tract can lead to translocation or leakage of *Candida* from the GI tract to the extracellular and/or vascular compartment. Therefore, infants with low flow states or gut ischemia and those who have undergone GI surgery are at greater risk for systemic invasion by *C. albicans*.[5,6] Such risk is further magnified if these infants are in a catabolic or malnourished state. Because of the immunosuppressive and catabolic effects of glucocorticoids, infants on such therapy are at additional risk for opportunistic fungal infections.

The advanced practice nurse can help reduce risk factors by limiting excessive and/or inappropriate antibiotic use, assisting staff in increasing the progression of infant nutrition from parenteral to enteral nutrition, and instituting nursing measures to wean infants from the ventilator. Advanced practice nurses can assist the NICU staff in recognizing infant readiness for feeding progression and ventilator weaning. Nursing actions that promote infant progression in the NICU decrease the occurrence of opportunistic fungal infections, because they are often seen in infants with prolonged NICU stays (for example, "late, late onset" sepsis).

Promote neonatal immun competence

The advanced practice critical care nurse can initiate many actions to pro-

mote neonatal immunocompetence. Research studies suggest that the more stressed an infant is, the less effective the infant's immune system.[23] This is most likely mediated through biological pathways that link the nervous, endocrine, and immune systems. Hormones secreted in response to stress produce various immunosuppressive effects.[39] Nursing actions that promote rest and reduce stress foster optimal development of the immune system as well as prevent any further impairment of immune function. To accomplish this, the advanced practice nurse should initiate a model of developmental care in the NICU to maintain the infant in a stable state. The staff should organize nursing care to promote optimum rest periods for the infant. Nurses should initiate care activities based on infant physical and behavioral cues.

The advanced practice nurse assists staff in providing an optimal NICU environment that promotes the development of the infant's sleep-wake cycle and other biological rhythms. This includes providing the infant with a period of time with dimmed lighting and a quiet environment. The staff should encourage family members to take part in the infant's care. Nurses can teach family members stress-reducing interactions they can provide for the infant. Such actions include tactile (holding, gentle massage), auditory (soothing talk, singing), and kinesthetic (rocking) stimuli, as appropriate to the infant's condition.

The advanced practice neonatal nurse can also demonstrate to the nursing staff ways to assess and promptly manage an infant's pain. Pain can trigger and/or amplify the stress response and stimulate secretion of endorphins, which have immunosuppressive effects.[39] In addition to ensuring adequate medication for pain, the advanced practice nurse can teach the staff ways to reduce pain from painful procedures, such as proper positioning; supporting the infant's position with back rolls or foot rolls while lying on the side; and offering comfort measures such as rocking, swaddling, or providing a pacifier.

Infant nutritional needs and management should be collaboratively determined by the advanced practice neonatal nurse and the neonatologist. Because the immune response is characterized by secretion of antibodies, cytokines, and rapid cell proliferation, adequate nutritional intake—especially of proteins—is essential. Furthermore, because of the associated risk of *Candida* infections in infants maintained on hyperalimentation and/or intralipids, it is important that the nurse continuously assess the infant's nutritional status and recognize cues that indicate either a readiness to initiate or advance enteral feedings or to slow or interrupt their progression. The advanced practice nurse can assist staff nurses in properly advancing the nutrition of infants in the NICU.

Maintain skin integrity

The advanced practice nurse needs to develop and implement protocols to maintain skin integrity of the infant in the NICU. The skin of the LBW infant, particularly of infants of less than 25 weeks' gestation, is highly fragile, and skin breakdown can occur with even gentle pressure.[40] Overall, the delicate and often transparent skin of the premature infant is more permeable and lacks bacteriostatic properties such as an acidic pH. In addition, the warm, moist environment of the NICU isolette facilitates the growth and colonization of fungi like *C. albicans*. As a result, *Candida* may readily colonize and easily attach to and invade the epithelial surfaces of the infant's skin. This commonly occurs in areas of skin folds, such as the axillae, groin, and neck.

Rigorous skin care protocols are a must for the infant in the NICU. This protocol may include use of skin barrier products under tape or other adhesive applications, a sterile nontoxic lubricant such as safflower oil for topical application, and hydrotherapy. In cases of severe skin breakdown, nurses can use a primary wound dressing such as Vigilon. Because of the high permeability of the premature infant's skin, which leads to absorption of topicals, nurses must exercise caution when using any product on a premature infant's skin.[40] It is important to frequently assess the infant's skin to identify any potential for breakdown.

Minimize antibiotic therapy

Because the development of commensal microflora along the mucosal surfaces is profoundly affected by antibiotic usage, the advanced practice nurse collaborates with the neonatologists, monitoring and minimizing antibiotic usage. She teaches the nursing staff how to inspect the infant's oral cavity for signs of oral candidiasis (thrush) and the anal area for perianal candidiasis. Mucosal surfaces of the GI, urinary, and respiratory tracts are prime portals of entry for invasive *C. albicans*. These sites are especially vulnerable in immature infants who are heavily exposed to *Candida* before the normal microbial flora of these mucosal surfaces develop. Excessive and inappropriate patterns of antibiotic use destroy the already insufficient microflora and place the infant at greater risk for superinfection and candidemia.[5,6]

Perform care of invasive devices

The advanced practice nurse ensures

that the care of invasive devices follows unit policy and that this policy reflects current opinion and research. Invasive lines and devices penetrate the barrier defense of the skin and/or mucous membranes and provide a portal of entry for microorganisms. Additionally, *C. albicans* has a propensity to adhere to and subsequently colonize on the plastic surfaces of such devices and under appropriate conditions can present a large inoculum to the infant.[24] Certain devices, such as a peripheral I.V. line, peripheral arterial line, percutaneous venous catheter, or central line, are generally covered with an occlusive dressing. Others, such as chest tubes or umbilical artery or venous catheters, usually are not. The advanced practice nurse can instruct staff regarding the routine inspection of device placement sites for signs of redness, drainage, or odors suggestive of infection.

Decrease environmental risk factors

To reduce the threat of nosocomial infection, nurse educators and advanced practice nurses can educate NICU personnel, family members, and visitors about infection-control policies. Staff are provided with guidelines that specify aseptic technique used when performing tasks of an invasive nature. Current protocols should be updated based on knowledge gained from nursing research.

The advanced practice nurse can instruct the NICU staff in preventing the spread of infection to other infants in the NICU. Although basic, the most important strategy to decrease infectious spread is proper hand washing. (Lapses in hand washing occur frequently, and to prevent such occurrences, the NICU staff must appreciate the importance of hand washing.[41]) An initial scrub is required for anyone entering the NICU environment, even if the person does not anticipate contact with an infant. In addition, hand washing is required between contact with infants. The nurse, as guardian of the critically ill infant's environment, should promote and update the unit's infection-control procedures as needed.

Recognize early signs of impending infection

The critical care nurse maintains continuous documentation of any subtle change in the infant's condition and therefore is, in many cases, the first to recognize the presence of sepsis. Regular assessment and surveillance of infants at greatest risk for early signs and symptoms of opportunistic fungal infections is a priority of neonatal care.

Any change in an infant's physical or behavioral state should raise the suspicion of fungal infection in infants with significant risk factors.

Early detection is generally difficult because the signs and symptoms of impending fungal infection—and of any septic condition—are usually vague. Yet, because these infections progress rapidly, early identification is fundamental to limiting their invasiveness. The nurse is pivotal in early detection because she is most familiar with the infant and will most likely recognize cues indicating early infection. Because of the insidious and nonspecific signs and symptoms of fungal infection, any change in the physical and/or behavioral state of an infant should raise the suspicion of fungal infection in infants who already have significant risk factors.

Signs and symptoms of invasive *Candida* infections may resemble bacterial sepsis or necrotizing enterocolitis. In many cases bacterial infection occurs coincidentally with systemic candidiasis. The clinical diagnosis of disseminated fungal infection is usually associated with one or more of the following: respiratory deterioration, abdominal distention, guaiac-positive stools, carbohydrate intolerance, candiduria, endophthalmitis, meningitis, abscesses, erythematous rash, temperature instability, lethargy, and hypotension.[5,13]

Multisystem involvement is very common, and signs and symptoms may reflect the specific organ system involved, again highlighting the difficulty the clinician is confronted with in the differential diagnosis of fungal infection. Diagnosis is also often delayed because of the inability to obtain a positive culture. It is generally accepted that the lack of positive bacterial cultures and the persistence of symptoms in LBW infants should always suggest the possibility of disseminated candidiasis.[5]

Despite the use of antifungal agents, mortality attributed to *Candida* infections can approach 60% in the smallest infants.[14,15] Infants who survive may suffer significant morbidity because of complications such as meningitis, renal abscesses, endophthalmitis, and osteomyelitis, which complicate their clinical course and compromise their quality of life.[6] In this regard, *Candida* infections contribute to the escalating costs of LBW infant care, because opportunistic *Candida* infections increase the length of hospital stay.[7] A more rapid and sensitive ap-

proach for diagnosis of neonatal systemic candidiasis and a more optimal treatment strategy for the infant with invasive candidiasis are major challenges for both critical care clinicians and researchers.

Institute therapy and evaluate response

Based on assessments, the nurse alerts the neonatologist to any suspicion of a fungal infection. Many cultures are negative even in the presence of fungal disease. If needed, administer appropriate antifungal therapy and treat the source of infection (for example, skin breakdown). Amphotericin B is the treatment of choice for neonates with disseminated fungal infections. It has fungistatic or fungicidal effects, which are contingent upon the resultant concentration in body fluids and the susceptibility of the organism. It is thought to act by binding to sterols in the fungus cell membrane, altering the membrane permeability, and allowing the loss of intracellular potassium.[13,38] However, because human cell membranes also contain sterols, it is theorized that amphotericin B may produce concomitant injury to cells other than the targeted fungal cells.[31] Keeping in mind this mechanism of lysis and cellular demise, the potential for renal or other tissue toxicity is clearly evident.

The action of amphotericin B on cellular sterols may result in diminished renal perfusion and glomerular filtration rates. Direct toxic effects of the drug may damage the tubular mucosa resulting in an augmented permeability to various electrolytes, hydrogen ions, and other solutes. Consequently, the neonate may exhibit a decrease in urine specific gravity and tubular reabsorption of sodium, renal tubular acidosis, and an increase in potassium excretion.[38] Infants receiving amphotericin B should be carefully monitored for signs and symptoms suggestive of such toxic effects, especially hypokalemia. The most commonly documented effects include oliguria, hypokalemia, acidosis, and elevated blood urea nitrogen (BUN), and creatinine levels. Other possible toxic effects include hepatotoxicity, hyperkalemia, thrombocytopenia, hypomagnesemia, fever, and thrombophlebitis of the peripheral I.V. site.

Neonatal dosing information varies slightly depending upon the source. McCracken and Nelson suggest a gradual increase in the dose delivered daily, starting with 0.25 mg/kg/day, to a maximum of 0.75 mg/kg/day.[42] According to Noerr, many neonatal pharmacology handbooks suggest amphotericin B beginning at 0.1 to 0.3 mg/kg/day and assessing the individual response of each infant being treated.[38] Overall, the range of recommended dosage for amphotericin B is 0.25 to 1 mg/kg/day as a daily infusion over 4 to 6 hours for 3 to 4 weeks.[13] Doses and duration of therapy are individualized based on the type of infection, the infant's immune response, and the infant's tolerance of the drug.[13,38]

Because of the potential for renal toxicity, careful assessment of renal function is a priority during amphotericin B therapy. Evaluation of fluid intake and urine output and periodic determination of urine specific gravity are routine. Blood levels of creatinine, BUN, and electrolytes (particularly potassium) are evaluated. Making sure the infant is well hydrated and reversing sodium depletion may prevent amphotericin-induced nephrotoxicity.

During the administration of the drug, vital signs should be monitored frequently. Nurses should observe for fever, tachycardia, cardiac arrhythmias, and hypotension.[43] Amphotericin B is never diluted or mixed with saline as this may lead to precipitation. Generally amphotericin B is mixed with a 5% dextrose solution. It is not compatible with total parenteral nutrition (TPN) and administration through the same line as TPN or any other medication known to be incompatible with amphotericin B is contraindicated. During administration, the bag should be protected from light. The manufacturer suggests that when using an in-line filter, the mean bore diameter should not be less than 1 micron. And finally, the rate of infusion is slow, ranging from 4 to 6 hours.[43]

Another treatment option for systemic candidiasis includes the combination therapy of amphotericin B and flucytosine. Flucytosine was initially developed in the late 1960s for treating cancer. However, it was soon noted to be effective as a systemic antifungal agent. While the precise mechanism of its antifungal activity is unknown, it is thought to be related to the conversion of flucytosine to fluorouracil in fungal cells. This competes with uracil and interferes with fungal RNA and protein synthesis. There appears to be a synergistic effect between amphotericin B and flucytosine against *Candida*, particularly for the treatment of *Candida* meningitis. Flucytosine appears to penetrate into the spinal fluid more readily than amphotericin B.[42] Flucytosine is generally not used alone to treat systemic fungal infection because several *Candida* strains are known to be resistant to this drug, and resistance can develop during therapy if used alone. On the other hand, others do not recommend flucytosine because there are no clinical data on its efficacy when used alone or in combination with amphotericin B for treatment of systemic candidiasis, and a rapid emergence of resistant *Candida* strains after flucytosine has been reported.[13]

Many of the untoward effects of

flucytosine are related to its potential for bone marrow depression resulting in neutropenia and thrombocytopenia. Infants receiving flucytosine are more prone to these types of complications if they already have a history of an underlying hematologic disorder. Flucytosine is rapidly and easily absorbed in the GI tract and is available for oral administration only. However, neonates with immature GI systems may not tolerate flucytosine therapy and this further limits its usefulness.[13,38]

The recommended dose of flucytosine for infants over 1 month is 100 to 150 mg/kg/day in four divided doses for 3 to 4 weeks, especially if severe infection or central nervous system involvement is evident.[37] Nursing interventions include most of the physiologic assessments associated with amphotericin B administration, along with additional assessments aimed at identification of GI disturbances such as abdominal distention, vomiting, increased residuals, or diarrhea; bone marrow suppression; and nephrotoxicity. Dosages are adjusted if renal insufficiency, neutropenia, or thrombocytopenia occur.[42]

CASE STUDY

Baby Girl Jones is a 24-week-old female, with a birth weight of approximately 700 grams (appropriate for gestational age). She was admitted to the NICU, where the advanced practice nurse assessed the prenatal history and alerted the primary care nurse to the infant's numerous risk factors for infectious disease. Baby Jones was born to a 29-year-old white female who came to the emergency department with sudden onset of labor, bulging amniotic sac, and complete cervical dilation. The pregnancy was complicated by a vaginal *Chlamydia* infection 2 weeks prior to delivery. The infection was treated with erythromycin. Prenatal care did not begin until the second trimester. Of particular significance, the nurse noted, the mother was febrile upon admission and her amniotic fluid was foul smelling. Antenatal glucocorticoids were administered in an attempt to enhance fetal lung maturation.

The infant was delivered by nonspontaneous vaginal delivery. The nursing staff assisted in aggressive resuscitation as the infant had no respiratory effort. She was intubated with a 2.5 cm endotracheal tube and was bagged on 100% oxygen until her blood oxygen saturation was above 90%. Apgars were 2 at 1 minute; 3 at 5 minutes; 6 at 10 minutes; and 7 thereafter. The baby received beractant (Survanta) in the delivery room. Initial ventilator settings were 20/4 (peak pressure/positive end-expiratory pressure) with a rate of 60%, and 100% fraction of inspired oxygen. The nurse admitting the infant to the NICU noted severe bruising over the scalp and extremities, fusion of the eyelids, and a three-vessel cord. Because of the septic risk, the advanced practice nurse consulted with the neonatologist and blood, cerebral spinal fluid, and urine cultures were obtained. The baby was started on ampicillin and gentamicin. However, all initial cultures were negative so antibiotic therapy was stopped after a 3-day course. The ultrasound of the head showed evidence of a Grade III bilateral intraventricular hemorrhage. An increase in head circumference and/or a bulging fontanel could indicate candidal meningitis as well as an intraventricular hemorrhage.

On day six, the infant's primary care nurse noted a marked change in the infant's clinical course. She observed that Baby Jones exhibited increasing restlessness and had increasing oxygen requirements. Baby Jones also demonstrated metabolic acidosis according to arterial blood gas measurements and had several episodes of severe bradycardia. The nurse carefully monitored the infant's I.V. fluids, but despite maintenance on a consistent concentration of 12% dextrose in her I.V. fluids, the infant's blood glucose level dramatically increased. Based on assessments and consultation with the neonatal nurse practitioner, a sepsis workup was initiated and antibiotics were restarted. Sepsis seemed certain when a left shift (an increase in the more immature leukocytes) was noted on the complete blood count. Two days later, cultures were positive for *Staphylococcus epidermidis*. The baby was then placed on vancomycin therapy for 10 days. Cultures were repeated and produced a negative result after completion of the vancomycin therapy. Feedings were not started due to the presence of a patent ductus arteriosus, which failed to close after six doses of indomethacin. At this time, the infant had a percutaneous venous catheter placed. Baby Jones was being main-

Preventive immunotherapy and innovative diagnostic procedures may help reduce the incidence of opportunistic fungal infections in critically ill infants.

tained on hyperalimentation and 10% intralipids, which further compounded her risk for fungal infection.

On day 20 the baby became severely neutropenic and thrombocytopenic, although repeated blood cultures proved negative. Inspection of the infant's skin revealed areas of breakdown, especially under the neck and axillae and in the groin. Despite an aggressive skin care regimen instituted by the neonatal nurse practitioner, the baby's skin problems became worse. Areas on the lower extremities required a primary wound dressing to cover exposed tissue. Blood cultures remained negative for organisms, including *Candida*. The nurse practitioner obtained several skin scrapings for culture. The cultures were positive for *C. albicans*. At this time, the baby was receiving almost daily transfusions of platelets for levels less than 20,000/nmm^3. The baby remained severely neutropenic. Eventually, blood cultures became positive for *C. albicans*, indicating that the infection was now systemic and required aggressive treatment. Amphotericin B therapy was initiated. The nurse practitioner discussed the infant's condition with the NICU staff and reinforced infection-control nursing actions to prevent spread of the infection.

After just 2 days on amphotericin B, the baby's skin began to improve, the platelet count exceeded 100,000/mm^3, and the white blood cell (WBC) count began to normalize. Treatment continued for 2 weeks, while the nurse closely monitored the infant for signs and symptoms of toxicity. The advanced practice nurse updated the NICU nursing staff on the clinical signs and symptoms of amphotericin B toxicity. Blood cultures were repeated with a negative result. With the candidemia episode and skin problems resolved, the baby was able to be taken to the operating room for ligation of her patent ductus arteriosus. Her recovery from the surgery was unremarkable. Shortly thereafter, enteral feedings were initiated and were well tolerated. No further episodes of sepsis occurred during her hospital stay. The neonatal nurse practitioner reviewed the baby's case with the nursing staff and provided a workshop to enhance the nursing staff's skills in risk assessment and management of opportunistic fungal infections.

In summary, Baby Jones had a history of significant risk factors for the development of fungal infection, which in her case originated in areas of skin breakdown. Because of the strong link between skin breakdown and fungal infections, the advanced practice nurse set the standards for skin care practices used in the unit. She educated the nursing staff to assess risk factors for fungal infection, which in this case included prematurity with LBW; maternal history of infection; maternal fever; foul-smelling amniotic fluid; antenatal glucocorticoids; intubation; vascular access lines; trauma to skin at birth; and the administration of antibiotics, hyperalimentation, and intralipids. The advanced practice nurse assisted her staff in recognizing signs of impending infection including increasing restlessness, increasing oxygen requirements, unstable vitals, carbohydrate intolerance, a leftward shift of the WBC differential, neutropenia, and thrombocytopenia. Unit workshops regarding hematologic changes indicative of impending infection were useful in educating the nursing staff about the immunologic response to invasive fungal infection.

FUTURE PERSPECTIVES

In order to decrease the incidence of opportunistic fungal infections in the critically ill infant, further development of prevention strategies and immune-enhancing interventions is needed.[2,3] The use of preventive immunotherapy is beginning and includes administering immune-activating cytokines such as G-CSF, GM-CSF, and IFN-γ in high-risk infants. Innovative procedures using nucleotide probes specific for *Candida* and use of DNA amplification procedures such as the polymerase chain reaction may provide more rapid and precise diagnosis of neonatal systemic candidiasis.[14] For treatment, a new advancement is the administration of amphotericin B in lipid complexes or liposomes. Liposome encapsulated amphotericin B has been tested in preterm infants with several fungal infections, and has been shown to be safe and effective. However, larger randomized clinical trials are needed to determine the most effective administration protocol.

In summary, morbidity, mortality, and health care costs due to opportunistic fungal infections are high. Future approaches to control fungal infections will require increased surveillance as well as the development of rapid, noninvasive diagnostic tests; monitoring of the development of resistance to antifungal agents; and research aimed at understanding, prevention, and control of fungal infections.[2]

CLINICAL RESEARCH QUESTIONS

The advanced practice nurse is in a pivotal position to provide leadership in the development of research projects that seek to prevent opportunistic fungal infections and improve the management of infants at risk for such infections. Examples of clinical research projects include determining:

1. What is the relationship between the use of pre- and postnatal

glucocorticoids on the incidence and severity of fungal infections?

2. How effective are skin care protocols on the colonization of the preterm infant's skin with fungi such as *C. albicans?*

3. How effective is a clinical risk scoring system in identifying infants most at risk for the development of opportunistic fungal infections?

SUMMARY

The incidence of opportunistic fungal infections by *C. albicans* has increased within recent years. Critically ill LBW infants are most vulnerable to disseminated fungal infection, and this risk is compounded by invasive care measures. Despite antimicrobial therapy, morbidity and mortality remain high. Development and implementation of preventive and management strategies by advanced practice nurses will decrease the incidence and poor outcome associated with invasive candidiasis. ■

KEY WORDS

Candida albicans, opportunistic fungal infections, low birth weight infants

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EDITOR'S NOTE

All names and insignificant characteristics have been changed for confidentiality. Any resemblance to a real person is coincidental.

Are we making progress in antifungal therapy?

Pietro Martino, MD, and Corrado Girmenia, MD

Contrary to the case with bacterial infections, progress in the diagnosis and treatment of invasive mycoses in cancer patients has been unsatisfactory. Amphotericin B deoxycholate has remained the drug of choice for severe invasive fungal infections for nearly 40 years. However, its infusion-related side effects, as well as its toxicity, may at times lead to dose reduction or early discontinuation of the treatment. The introduction of the new triazoles, fluconazole and itraconazole, has improved the therapeutic chances against several fungal infections; however, the need for a broad-spectrum drug in empiric antifungal therapy, the emergence of fluconazole-resistant *Candida* species, and the limitations of itraconazole in terms of speed of action and erratic oral absorption represent important limitations. Recently, laboratory and clinical research has been directed at the develoment of new formulations of older classes of antifungals, the introduction of new classes of antifungals, and the use of immunomodulation associated with antifungal therapy. This paper reviews the more recent advances in the treatment of fungal infections in cancer patients.

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Abbreviations

AmB	amphotericin B
AmBd	amphotericin B deoxycholate
AmBd-LE	amphotericin B deoxycholate plus lipid emulsion
G-CSF	granulocyte colony-stimulating factor

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In the previous review by Walsh *et al.* [1] on recent progress in the management of fungal infection in cancer patients, published in 1992 in *Current Opinion in Oncology*, the old amphotericin B deoxycholate (AmBd) was still considered the drug of first choice for the treatment of invasive mycoses. The promising role of fluconazole and itraconazole in the treatment of candidosis and aspergillosis, respectively; the possibility to improve amphotericin B (AmB) therapy by administering higher doses of the less toxic lipid formulations of the drug; and the possible usefulness of growth factors were predicted to be the avenues of real progress in antifungal treatment strategies in the next few years.

This paper reviews the more recent advances and verifies the expected progress in therapy of fungal infections in cancer patients.

New experiences with old drugs

Amphotericin B versus fluconazole in the treatment of *Candida* infections and in empiric antifungal therapy

Despite the increasing use of the triazoles fluconazole and itraconazole as therapy for systemic mycoses, direct comparison of the efficacy of these drugs with that of AmBd in the treatment of life-threatening infections is still lacking. This lack is probably due to the difficulties of carrying out randomized studies with an adequate number of patients with documented and homogeneous fungal infections. In the last years, however, AmBd and fluconazole have been compared in various large studies for the treatment of candidemia, a common fungal infection of rather easy documentation. In a multicenter randomized trial of 206 non-neutropenic patients with various underlying diseases, fluconazole and AmBd were not significantly different in their effectiveness in treating candidemia [2••]. However, in this study, a central venous catheter was considered to be the source of candidemia in most patients, and an invasive infection was documented in only a minority of cases. Therefore, the question of whether treatment with fluconazole and treatment with AmBd in patients with candidemia and deep-seated infection are comparable remains unanswered [3].

Both in a large, multicenter, prospective, observational study, and in a matched cohort study of cancer patients, fluconazole was shown to be better tolerated and as efficacious as AmBd in the treatment of candidemia, even when the data were stratified by risk factors for mortality [4•,5•].

In a multicenter, prospective, randomized clinical trial, intravenous fluconazole was by far less toxic and as effica-

cious as AmBd in the empiric treatment of unexplained fever resistant to antibacterial therapy in neutropenic cancer patients [6]. However, patient numbers were too small to allow definitive conclusions about efficacy, and the use of fluconazole for this indication therefore remains experimental.

Antifungal prophylaxis

Previous experiences in the prophylactic use of fluconazole showed that this triazole is effective in reducing both invasive and superficial *Candida* infections, but without survival improvement, in patients undergoing bone marrow transplantation. More recently, a randomized, double-blind, placebo-controlled trial newly assessed the efficacy and toxicity of 400 mg/day of fluconazole in preventing fungal infections after marrow transplantation [7••]. In this experience, prophylactic fluconazole was safe, reduced systemic and superficial fungal infections, and improved survival at day 110 after marrow transplantation.

Infection by resistant fungal pathogens is an emerging cause of failure of antifungal prophylaxis; however, there are recent reports of breakthrough fungemias by susceptible isolates in patients with indwelling central venous catheters [8•,9•,10]. Antifungal prophylaxis could be clinically unhelpful in preventing fungal localization into the central venous catheter, in keeping with the observation that antifungal therapy is ineffective against catheter-related fungemias unless the catheter is removed. Owing to the frequent use of venous devices in cancer patients, the possible role of the venous catheter in the failure of antifungal treatment should be always considered in the clinical evaluation of cancer patients with blood stream fungal infections.

Fluconazole and mold infections

Fluconazole is a selective antifungal drug with no activity against molds at the standard dosages. A trial of high doses (800–2000 mg/d) of fluconazole was conducted in 28 cancer patients with mold infections [11•]. Fluconazole was well tolerated at total daily doses up to 1600 mg, but its activity seemed to be limited.

Combination therapy

With the aim of improving cure rates and reducing the dose, and hence the toxicity, of AmBd, the use of combination antifungal therapy has been considered for several years.

The combination of AmBd and flucytosine has been employed in the treatment of cryptococcal meningitis and has frequently also been extended to other yeast infections. Recently, the potential role of other combinations was investigated. *In vitro* studies show that fluconazole and flucytosine interact favourably against a large number of isolates of *Cryptococcus neoformans* [12], which suggests that prospective trials in patients with cryptococcal meningitis should be instituted.

Despite some evidences of *in vitro* antagonism between AmBd and azoles, these combinations have been explored in animal models. Fluconazole was more active than AmBd against experimental murine trichosporonosis, but the activity of the combination of the two drugs appeared to be superior to that of either agent alone [13]. In a study of murine invasive candidiasis, combination therapy with AmBd and fluconazole was superior to therapy with fluconazole alone and at least as efficacious as therapy with AmBd alone, thus demonstrating the absence of *in vivo* antagonism [14].

These experiences seem to suggest that the combination of AmBd plus triazoles is not antagonistic *in vivo*, and its possible role in the treatment of invasive *Candida* infections in humans should be thoroughly evaluated.

Role of antifungal susceptibility testing in the management of fungal infections

The emergence of fungal infections that are resistant to the antifungal drugs, particularly the triazoles, represents one of the most important problems in the management of mycoses; thus, the surveillance of antifungal susceptibility is needed. On the basis of pharmacokinetic and clinical evaluations, a tentative predictive model for identifing the correlation between treatment with fluconazole and itraconazole minimal inhibitory concentrations and the outcome of candidal infections has been proposed [15••]. The authors conclude that breakpoints for antifungal susceptibility testing should not be considered to absolutely predict successful treatment of infections caused by *in vitro* susceptible isolates, but that resistance *in vitro* seems to represent a valid criterion for predicting therapeutic failure.

New formulations of old drugs: lipid formulations of amphotericin B

For the past 10 years, evidence has existed that encapsulating AmB into liposomes or binding AmB to other lipid carriers is associated to a significant reduction of AmB toxicity. Consequently, the new formulation of AmB promise to have a higher therapeutic index than conventional AmBd. However, only in recent last years have laboratory and clinical trials been reported that evaluate the toxic activity and the therapeutic index of the lipid formulations of AmB. Clinical advantages in comparison with AmBd are still under evaluation. Three lipid formulations of AmB are now under clinical investigation: (1) AmBisome (Nexstar, San Dimas, CA); (2) AmB lipid complex (ABLC; Abelcet, Liposome Company, Princeton, NJ); and (3) AmB colloidal dispersion (ABCD; Amphocil, Sequus Parmaceuticals, Menlo Park, CA).

Recently, AmBd mixed in a lipid emulsion (Intralipid 20%) was used in several centers in the Americas and

Europe with the purpose of obtaining the same efficacy and low toxicity of the previous lipid formulations of AmBd.

AmBisome is the only liposomal preparation of AmB. In an animal model of systemic candidiasis in leukopenic mice, AmBisome at doses of 5 mg/kg, 11 mg/kg, and 29 mg/kg were not effective, partially effective, and completely effective, respectively [16]. It seems that a maximal reduction of AmB toxicity results in a concomitant loss of antifungal activity and efficacy.

The clinical efficacy of liposomal AmB in the treatment of fungal infections in cancer patients was investigated in noncomparative, retrospective experiences [17•,18]. In a single-center experience, AmBisome was used for the treatment of 133 suspected or confirmed fungal infections in neutropenic patients [17•]. The drug was well tolerated, even at the dose of 5 mg/kg/day, and no significant renal impairment was observed. The mycosis was resolved with AmBisome treatment in 81 (61%) of the cases. In particular, a complete or excellent partial resolution of the signs of infection was obtained in 32 of 57 (56%) patients with proven or suspected aspergillosis, including 6 of 11 patients who failed to respond to a previous treatment with AmBd.

The response to AmBisome treatment in this experience was high, taking in account that in several cases the drug was used as a salvage therapy. However, absolute neutrophil count was found to be significantly higher in responder patients during the first 14 days of treatment. Thus, the separate roles of AmBisome and of neutrophil count recovery in the resolution of the infection could not be assessed in this unconrrolled study.

A double-blind study of prophylaxis among 76 patients undergoing bone marrow transplantation compared AmBisome (1 mg/kg/day; 36 patients) with placebo (40 patients) [19]. The side effects, the incidence of suspected or autopsy-proven invasive mycosis, and the overall survival for patients who had received placebo was similar to that for patients who were treated with AmBisome.

Preliminary results from two prospective, randomized studies comparing AmBisome treatment with traditional AmBd treatment in adults and pediatric patients with hematologic malignancies seem to show a higher efficacy of AmBisome in the treatment of suspected fungal infections. Complete data for a full evaluation of the studies are needed.

ABLC is a concentration of ribbon-like structures of a bilayered lipidic membrane. In a recent experience of six patients with invasive fungal infections, large cumulative doses (22.3–73.6 g) of ABLC over a mean of 53.8 weeks were well tolerated and none of the patients experienced ABLC-associated toxic effects that would necessitate discontinuation of the treatment [20].

The largest published series of patients treated with ABLC comes from the North American experience of 800 patients enclosed in a open-label emergency-release protocol [21•]. The criteria for the enrollment in the study were failure of previous systemic antifungals, nephrotoxicity or severe acute toxicity with AmBd, underlying renal disease, or nephrotoxicity due to other drugs. ABLC was administered at a dose of 5 mg/kg/day for at least 4 weeks. In the preliminary analysis of the first 228 treatments, the overall clinical response rate was 69% (126 of 183 patients evaluable for response). Treatment with ABLC was well tolerated, and in patients with previous renal failure the mean serum creatinine levels declined during therapy. In this preliminary study, ABLC seemed to be effective in the treatment of invasive mycoses, and the response rate was similar in different categories of patients according to type of infection, underlying disease, and degree of neutropenia. These data seem to demonstrate the good tolerability and low toxicity of ABLC. In contrast, the extreme variety in the types of infections and in the categories of patients, and the lack of a control population do not permit any definitive evaluation of efficacy.

The results of randomized prospective trials comparing ABLC and AmBd in the treatment of invasive mycoses in cancer patients have been presented as preliminary communications in international congresses. Only a randomized study comparing the two formulations of AmB in the treatment of cryptococcal meningitis in patients with AIDS has been published [22••]. ABLC showed comparable activity and less hematologic and renal toxicity than AmBd.

ABCD is composed of disklike structures of cholesteryl sulfate complexed with amphotericin B. The pharmacokinetics, safety, and efficacy of ABCD have been extensively studied in animal models [23,24•,25]. In a model of experimental pulmonary aspergillosis in persistently granulocytopenic rabbits, the antifungal activity of ABCD was directly related to the increasing dosage. Microbiologic clearance in AmBd-treated (1 mg/kg/day) control rabbits was similar to that in rabbits treated with 5 or 10 mg/kg/day of ABCD. However, as determined by extension of pulmonary infection and by survival, ABCD at 5 mg/kg/day was more effective than AmBd at 1 mg/kg/day [23,24•].

These data are in keeping with a pharmacokinetic study in rats [25], which showed that, after administration of ABCD and AmBd at an equal dose (1 mg/kg/day), AmB concentration in plasma and most tissues was lower for the ABCD dose, whereas plasma AmB concentration-time

profiles afrer adminisrration of 5 mg/kg ABCD were similar to those found afrer administration of 1 mg/kg AmBd. Only AmB concentrations in rhe liver were higher in rats treated with 1 mg/kg ABCD.

The pharmacokinetics of ABCD, irs effect on creatinine clearance, and its efficacy were evaluared in a phase I dose-escalaring srudy (from 0.5 to 8 mg/kg/day) in 75 patients with systemic fungal infections who underwent bone marrow ttansplantation [26,27••]. The estimated maximum tolerated dose was 7.5 mg/kg. The complete or partial response rate was 52%.

In an open-label ttial, 168 patients with invasive mycoses were treated with ABCD at doses up to 6 mg/kg/day [28]. The patients had failed ro respond to a previous AmBd rreatment, had experienced treatment-limiting toxic effects of AmBd, or had pre-existing renal impairment. Complete clinical response or improvemenr was observed in 49% of the patients (48 of the 97 evaluable cases) and ABCD was associated with little renal toxicity. The response rate was similar whether parients had received more or less than 500 mg of AmBd prior to ABCD therapy. Clinical efficacy appeared to correlate significantly with the degree and persistence of neutropenia, and most of complete tesponses occutred in patients with end-of-treatment neutrophil counts of 500/μL or greater. Therefore, it is difficulr to assess, from this experience, the contribution of recovery from neutropenia to the response to ABCD.

Several experiences on the use of AmBd plus lipid emulsions 20% (AmBd-LE) have been reporred in both Europe and United Srates [29•,30–32]. In a French experience, AmBd diluted in either 5% dextrose or in 20% lipid emulsion (Intralipid) were randomly compared in the treatmenr of 42 patients with hematologic malignancies requiring antifungal therapy [29•]. Patienrs treared wirh AmBd-LE experienced significantly lesser AmBd infusion-related chills and nephrotoxicity. No difference was found between the two groups in potassium and sodium supply. With regard to efficacy of the two treatmenrs, no difference in survival was observed, although rhe few cases (only three) of documented fungal infections and the small number of parients enrolled in the study did nor permit any assessmenr of efficacy. In contrast with the previous encouraging results, the use of AmBd-LE is criricized by some authors who showed rhat AmBd does nor mix well wirh the fat emulsion Intralipid [33–35]. The formation of particles in rhe solurion requires filrration, which could also remove AmBd bound to these aggregates, thus allowing administration of an unpredictably lower dose rhan inrended [35].

New antifungal drugs

New azoles and new classes of antifungal agents are under investigation in *in vitro* and *in vivo* experimental studies, and phase I and II clinical studies are in progress for some of them. The broad spectrum of acrion, the favorable pharamacokinetics, and the low toxiciry are rhe primary expected characteristics of these drugs. The newer triazoles D0870, SCH 51046, SCH 51048, SCH 56592 and voriconazole (UK-109496) are more porent in animal models of invasive fungal infecrion and in *in vitro* srudies rhan fluconazole and irraconazole [36–42,43••]. D0870 was found to have low acrivity against murine aspergillosis [38]. Voriconazole was 10 to 100 times more potent than fluconazole in a *in vitro* study against *Candida* species [43••]. Thanks ro its favorable pharmacokinetics and ro irs broad spectrum of antifungal activity, including activity against *Candida*, *Aspergillus*, and *Cryptococcus*, voriconazole seems to be a promising antifungal drug, particularly in the setting of infections due to resistant fungal species.

New classes of antifungals that will potentially be available in the furure are under invesrigation. Data from preclinical evaluarions show that the pneumocandins should be safe, broad-spectrum fungicidal agents and potent parenteral therapeutic agents for disseminated *Aspergillus* and *Candida* infections [44–47]. The main limitation of the spectrum of pneumocandins seems to be the lack of coverage for *C. neoformans* [45]. Pradimicin and nikkomycin are other new classes of broad-spectrum antifungals under clinical developmenr [48,49].

New applications of growth factors

Profound, persistent neutropenia is a major risk factor for invasive fungal infections, and tecovery of neutropenia is a determinant in the prognosis. As shown in experimental animal models [50] and in a preliminary study [51•], the administrarion of colony-stimulating facrors combined wirh antifungal drugs could possibly improve the treatment of invasive fungal infections in neutropenic cancet patients.

Granulocyre colony-stimularing factor (G-CSF) has been successfully employed as adiuvant therapy in some cases of invasive zygomycosis or fusariosis [52–55], which are opportunistic fungal infections associated with a particularly high mortaliry rate in patients with persistent neutropenia. The improvement of clinical manifesrations was closely related ro neurrophil recovery, probably favored by G-CSF administration.

G-CSF has been also administered to leukocyte donors to markedly increase rhe leukocyte yields. Some patients with fungal infections thar do not respond ro adequare antifungal therapy, responded to rhe treatment afrer being rransfused with polymorphonuclear neutrophil concentrates collected from G-CSF-primed donors [56••]. However, it was difficult to prove rhat the clinical response was directly due ro G-CSF-stimulated whire blood cell transfusion.

Although encouraging, the published experiences on the use of growth factors as adjuvant rherapy of invasive

mycoses are only anecdotal reports. Randomized clinical trials are needed to clarify the role of these expensive drugs in the strategies of antifungal therapy.

Conclusions

Over the last years, there has been a strenuous laboratory and clinical research activity aimed at identifying the therapeutic approach of severe fungal infections. Since the previous review in 1992 by Walsh *et al.* [1], the following progress in the various fields of antifungal therapy strategies have been achieved:

New experiences with old drugs

Some indications for the use of the triazoles are now more codified. Fluconazole could be a valid alternative to AmBd in the treatment of candidemia, but its role against severe invasive infections remains uncertain. The possible enhanced antifungal activity of combination therapy should be investigated.

New formulations of old drugs

The safety of lipid formulations of AmB has been widely demonstrated. To date, underlying renal disease and nephrotoxicity due to AmBd or other drugs represent the only acceptable indications of the use for lipid formulations of AmB. Further data on the efficacy of lipid AmB preparations compared with AmBd are needed, also given the high costs of these agents.

New antifungal drugs

The new triazoles and the new classes of antifungal agents could represent a valid alternative, owing to interesting antimicrobial and pharmacokinetic characteristics, but their safety and efficacy have not been clinically investigated. At this moment, we cannot predict their real contribution to improving the treatment of fungal infections in immunocompromised patients.

New applications of growth factors

The use of growth factors as adiuvant therapy for invasive mycoses in neutropenic patients seems to be promising, but it has been until now only anedoctally reported.

We can conclude that we do progress in the field of therapeutic strategies of fungal infections in cancer patients; however, for 40 years, AmBd has been and continues to be the "gold standard" of empiric antifungal therapy and of treatment of most documented fungal infections.

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Genetics

Exhibit G

Dominant negative selection of heterologous genes: Isolation of *Candida albicans* genes that interfere with *Saccharomyces cerevisiae* mating factor-induced cell cycle arrest

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ABSTRACT **We have used a genomic library of *Candida albicans* to transform *Saccharomyces cerevisiae* and screened for genes that act similarly to dominant negative mutations by interfering with pheromone-mediated cell cycle arrest. Six different plasmids were identified from 2000 transformants; four have been sequenced. One gene (*CZF1*) encodes a protein with structural motifs characteristic of a transcription factor. A second gene (*CCN1*) encodes a cyclin homologue, a third (*CRL1*) encodes a protein with sequence similarity to GTP-binding proteins of the RHO family, and a fourth (*CEK1*) encodes a putative kinase of the ERK family. Since *CEK1* confers a phenotype similar to that of the structurally related *S. cerevisiae* gene *KSS1* but cannot complement a *KSS1* defect, it is evident that dominant negative selection can identify proteins that complementation screens would miss. Because dominant negative mutations exert their influence even in wild-type strain backgrounds, this approach should be a general method for the analysis of complex cellular processes in organisms not amenable to direct genetic analysis.**

Complementation of defective genes in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has been used to identify functionally related or homologous genes from a number of organisms, including humans (e.g., refs. 1–3). The complementation approach is powerful, but it has limitations since heterologous proteins may often not be able to fully complement a phenotype and replace a missing activity, even if expression levels are high. However, because in some situations high-level expression of partially defective proteins can interfere with the function of the corresponding wild-type protein, some heterologous genes may act as novel examples of dominant negative mutations (4). Expression of such foreign proteins could potentially interfere with cellular functions in different ways; for example, the foreign protein could be (*i*) a dysfunctional component of a multimeric complex that inactivates the entire complex, (*ii*) a negatively acting element that cannot be shut off, or (*iii*) a component that competes for some limiting cofactor or metabolite.

We have tested the efficiency of a dominant negative selection scheme for the identification of heterologous proteins that interfere with the mating response pathway in *S. cerevisiae*. This pathway can be considered a model for signal transduction systems (for review, see refs. 5 and 6), and it provides a very good assay for a dominant interference selection because the pathway has many potential targets and a sensitive positive selection (pheromone resistance) to allow detection of interfering functions. This pathway has been extensively studied by more conventional means, and the selection of strains insensitive to pheromone-mediated cell cycle arrest has proved to be an important tool for the analysis of the pheromone response pathway. Recessive mutations leading to pheromone resistance (7, 8) have identified genes encoding the α-pheromone receptor (9, 10), the pheromone response G-protein β subunit (11), two protein kinases (12, 13), a transcription factor (14), and a product apparently involved in regulating cyclin activity (8). Dominant mutations leading to pheromone resistance led to the isolation of a G_1 cyclin (15). In addition, overexpression of *S. cerevisiae* genes that reduce pheromone sensitivity has allowed the identification of a G-protein α subunit (16) as well as the KSS1 protein kinase (17).

We have used *C. albicans* as the source of potential interfering sequences. A variety of *C. albicans* genes can be expressed in *S. cerevisiae*, yet the *C. albicans* sequences diverge significantly from their *S. cerevisiae* homologs at the protein level (18). In addition, although it does contain signal transduction components, including a putative G-protein α subunit (19), *C. albicans* is apparently an asexual diploid organism (18) and thus does not contain a pheromone response pathway analogous to that in *S. cerevisiae*. We anticipated that *C. albicans* could provide a source of proteins structurally related to elements of the *S. cerevisiae* mating response pathway, yet not normally functioning in a mating response pathway; overexpression of such proteins could potentially interfere with proper pheromone-mediated cell cycle arrest. Our results show that this selection of interfering functions using libraries from heterologous organisms is efficient and suggest that this approach could provide a strategy for the identification of genes acting in a variety of cellular pathways in otherwise genetically intractable organisms.[†]

MATERIALS AND METHODS

Recombinant Techniques. Standard protocols were used for yeast and *Escherichia coli* (20–22) and for DNA sequencing (23).

Strains and Plasmids. *S. cerevisiae* strains used were M200-6C (*MATa ura3 ade1 sst1 sst2 ilv3*) (24), EY965 (*MATa ade2 his3 leu2 trp1 ura3 can1 fus3::LEU2 kss1::HIS3*), a gift from E. Elion (Harvard University), and YNN19 (*MATa ura3 leu2 his3 trp1 lys2 fus1::lacZ LEU2*), a gift from K. Matsumoto (Nagoya University). Strain 1316-5C (*MATa cln1Δ cln2Δ cln3Δ sst1 trp1 ura3 ade1 his2 leu2::pGAL CLN3–LEU2*), with deletions of the three *CLN* genes and expressing *CLN3* under galactose-inducible control, was from F. Cross (Rockefeller University). Plasmids other than those identified in the library screening were YEp352, a *URA3* 2-μm vector containing the M13mp18 polylinker (25), and pBC50,

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Abbreviation: aa, amino acid(s).

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[†]The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M76585 (*CEK1*), M76586 (*CZF1*), M76587 (*CCN1*), and M83991 (*CRL1*)].

which contains *KSS1* under control of the *GAL1* promoter and was provided by W. Courchesne (University of Nevada).

Assays. Pheromone treatment was as described (11) with α-factor. β-Galactosidase activity was assayed essentially as described (21).

RESULTS

Isolation of Interfering Sequences. A high-copy library of genomic sequences from *C. albicans* strain WO1 was generated from DNA partially digested with *Sau*3A1, size-fractionated to 5–10 kilobases, and cloned into the *Bam*HI site of YEp352. The library contains 30,000 independent plasmids, of which half contain inserts (C. Boone, personal communication). This library was introduced into M200-6C, a pheromone-supersensitive strain of *S. cerevisiae* defective in both *SST1* (gene required for α-factor degradation) and *SST2* (gene required for adaptation to pheromone). Transformants were then screened on plates spread with 5 μg of synthetic α-factor. Use of the highly pheromone-sensitive *sst1 sst2* double mutant host strain allowed even minor increases in pheromone resistance to be detected.

Approximately 2000 transformants were tested for those which conferred enhanced resistance to the *S. cerevisiae* α pheromone. Twelve resistant colonies were detected, but 3 were not dependent on the plasmid for the resistance and thus presumably represented sterile mutants, which can arise frequently (24). The remaining transformants contained plasmids that could be retransformed into M200-6C to reestablish the pheromone resistance. These 9 plasmids were mapped and subcloned to define the region that interfered with the pheromone-mediated cell cycle arrest. Two plasmids were identical (M152p8 and p8.2) and contained a region that overlapped with that of a third plasmid (M161p11). Another region conferring resistance was identified by two overlapping clones (M153p11 and M162p19), and the regions contained in four other plasmids were unique (M157p4, M163p21, M166p16 and M167p11).

The behavior of the 6 unique plasmids was further characterized. The plasmids enhanced the pheromone resistance of strain M200-6C to varying degrees. Strain M200-6C transformed with the vector plasmid YEp352 was unable to grow in the presence of α-factor (Fig. 1*A*). In contrast, transformants containing plasmids M152p8 and M153p11 grew well, those with M163p21, M166p16 and M157p4 grew moderately well; and those containing M167p11 grew marginally better than the controls. None of the plasmids encoded α-factor-degrading activity (barrier activity) (27), and thus none of the sequences complemented the *sst1* mutation (data not shown).

The response of each transformant was also analyzed by microscopic examination of the pheromone-treated cells (Fig. 1*B*). The transformants containing plasmids M152p8 and M153p11 did not undergo the morphological changes in the presence of α-factor that characterized cells containing the control plasmid YEp352. Transformants with plasmid M166p16 had many cells that underwent morphological changes in response to pheromone, but they also contained frequent cells that were dividing at a smaller than normal size. The other plasmids did not dramatically alter the behavior of the cells; after pheromone treatment the cellular morphology was similar to that of the strain containing YEp352.

We also examined the effect of the interfering plasmids on the induction of *FUS1*. The expression of this gene is highly induced by pheromone treatment and thus serves as a good marker for the transcriptional response to pheromone. Strain NYY19a, which contains a *fus1*::*lacZ* fusion and thus expresses β-galactosidase under control of the *FUS1* promoter, was transformed with various plasmids. β-Galactosidase expression was measured with and without α-factor addition. None of the plasmids dramatically interfered with *FUS1* induction: plasmids M153p11 and M157p4 reduced *FUS1* induction to about 80% of the control, whereas M167p11 reduced it to 70% of the control, and the other plasmids tested had no effect (data not shown).

Characterization of Interfering Plasmids Containing *CEK1*. Initial restriction analysis suggested that two of the interfering plasmids, M152p8 and M161p11, defined the same gene. Comparison of the two sequences revealed that the plasmids encoded proteins that were identical except for the size of a polyglutamine tract near the N terminus of the putative interfering protein. This polymorphism may reflect differences in the two potential alleles from the diploid source of the *C. albicans* library DNA. After a glutamine/alanine-rich N-terminal 60 amino acids, the rest of the protein was a putative protein kinase with a high level of sequence identity to *S. cerevisiae* kinases *FUS3* (28) and *KSS1* (17) and to a variety of mammalian kinases of the ERK (extracellular signal-regulated kinase) class (29) (Fig. 2). Subclones that interrupted the kinase coding sequence destroyed the ability to confer pheromone resistance, confirming that the putative kinase encoded the interfering function. We have designated this gene *CEK1*, for *C. albicans* ERK-like kinase.

Because of the significant sequence similarity with the *KSS1* and *FUS3* genes of yeast, and the observation that these two genes function in a redundant fashion in the mating process (30), we asked whether *CEK1* could substitute for *FUS3/KSS1* function. Transformation of a *fus3 kss1* strain, EY965, with *CEK1* did not reestablish mating competence. However, introduction of plasmid pBC50, which contains *KSS1* gene under galactose control, allowed mating of strain EY965 in a galactose-dependent manner. In addition, pBC50 conferred pheromone resistance on M200-6C in a galactose-

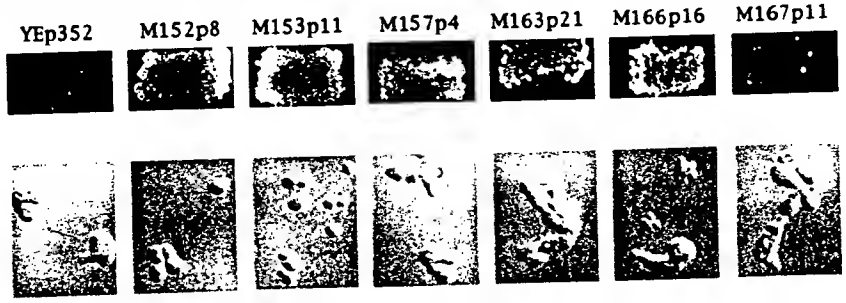

FIG. 1. Pheromone response of M200-6C cells containing various plasmids. (*A*) Growth of transformants on plates spread with 5 μg of α-factor. Patches of cells grown on uracil-free plates were replicated to uracil-free plates with α-factor. Growth after 2 days of incubation at 30°C is shown. (*B*) Cellular response to α-factor. The same transformants shown in *A* were incubated overnight in liquid uracil-free medium containing α-factor at 1 μg/ml, and the cells were photographed with a Leitz Aristoplan microscope using Nomarski optics. Cells containing the control plasmid YEp352, as well as plasmids M157p4, M163p11, and M167p11, exhibited extensive morphological changes in response to pheromone, while those containing plasmids M152p8 and M153p11 exhibited essentially no response. Cells with plasmid M166p16 were often tiny, although frequent responding cells could be detected. (×320.)

dependent manner, indicating that both *KSS1* and *CEK1* confer similar pheromone-resistant phenotypes when overexpressed (data not shown).

Characterization of a Plasmid Containing *CCN1*. The second plasmid analyzed, M166p16, conferred significant pheromone resistance on strain M200-6C, and the cellular morphology of the transformants showed that many cells were dividing at a reduced size (Fig. 1). The gene responsible encoded a region with significant similarity to the "cyclin box" elements of a variety of cyclin genes. The greatest similarities were with the *Sch. pombe* G_2 cyclin cdc13 and G_1 cyclin puc1, as well as the *S. cerevisiae* G_1 cyclin CLN3 (Fig. 3). The *C. albicans* gene product showed identity with puc1 of *Sch. pombe* and CLN3 of *S. cerevisiae* at most of the residues that define the G_1 cyclin family (33). Thus, it appears to be a G_1 cyclin. We have designated this gene *CCN1*, for *C. albicans* cyclin. Because the reading frame extends through the end of the original clone, the gene in plasmid M166p16 does not encode the C terminus.

To assess whether *CCN1* could function as a replacement for the CLN family of *S. cerevisiae* cyclins, we introduced plasmid M166p16 into strain 1316-5C, which has deletions of *CLN1*, *CLN2*, and *CLN3*. This strain is viable only on galactose medium due to the expression of *CLN3* under the control of the *GAL1* promoter; it cannot grow on medium containing glucose as a carbon source. Transformants of this strain containing plasmid M166p16 could proliferate on glucose-containing medium, although the cells were dividing at a smaller than normal size, whereas transformants containing the control plasmid YEp352 could not grow on glucose medium. Thus *CCN1* can supply a G_1 cyclin function in *S. cerevisiae* (data not shown).

Characterization of a Plasmid Containing *CZF1*. Plasmid M157p4 conferred moderate pheromone resistance on strain M200-6C (Fig. 1). It contained an open reading frame without overall strong similarity to any sequence in the data base, but two distinctive motifs could be discerned (Fig. 4). The central part of the molecule was rich in glutamine residues, and the bulk of these residues defined four clusters of about 10 aa spaced at intervals of ≈50 aa. Glutamine-rich regions are found in a number of regulatory proteins (26, 34) and may be involved in transcriptional activation. The C terminus of the protein encoded by this interfering gene contained a cysteine-rich region similar to the zinc finger elements encoded by *LAC9* of *Kluveromyces lactis* (35) and by *GAL4* (36) and *HAP1* (37) of *S. cerevisiae*. We have designated this gene *CZF1*, for *C. albicans* zinc finger protein.

Overall the structure of CZF1 is reminiscent of that of a transcription factor. Deletion of a large internal region of the protein between the two *Sac* I sites (Fig. 4), which included the glutamine-rich stretches, still allowed interference with the signal transduction pathway, while deletion of the C-terminal zinc finger domain totally eliminated the interference. Thus the interference comes from the putative zinc finger domain and not the other parts of the molecule.

Characterization of a Plasmid Containing *CRL1*. Plasmid M167p11 conferred weak pheromone resistance on strain M200-6C (Fig. 1). This plasmid encoded a putative gene product with high sequence similarity to a variety of small GTP-binding proteins (Fig. 5). The greatest sequence identity was with proteins of the *RHO* class of genes, so we have designated this sequence as *CRL1*, for *C. albicans* RHO-like protein. This protein of 346 aa is considerably larger than other members of the family, which are typically about 200 aa. This difference in size is due to an N-terminal extension of 80 aa, together with a block of 120 aa in CRL1 just before the C terminus, which is only 60 aa in the other RHO proteins. These two regions do not contain any identifiable motifs.

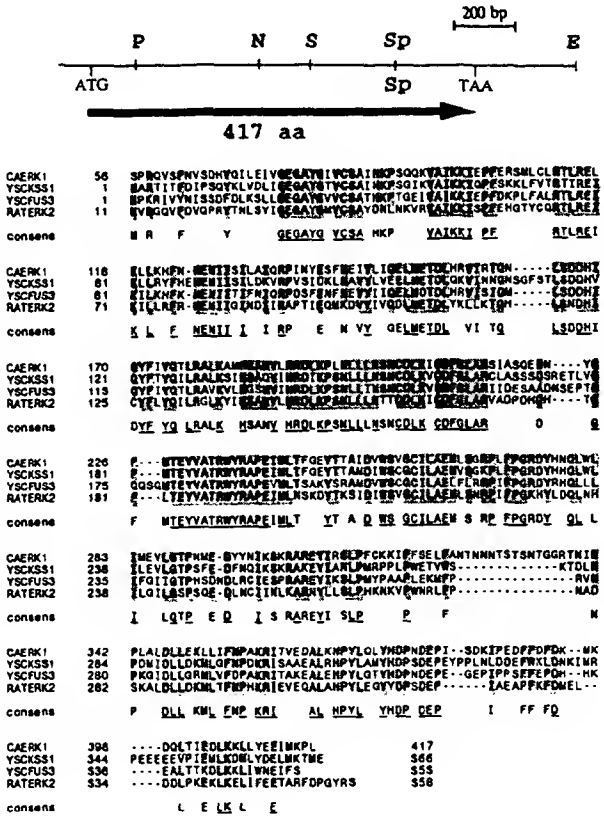

FIG. 2. The *C. albicans CEK1* gene. (*Upper*) Restriction map. E, *Eco*RI; N, *Nsi* I; P, *Pst* I; S, *Sac* I; Sp, *Spe* I; aa, amino acids; bp, base pairs. This gene maps to *C. albicans* chromosome 4. (*Lower*) Comparison of CEK1 (CAERK1) with the KSS1 (YSCKSS1) and FUS3 (YSCFUS3) kinases of *S. cerevisiae*, and the rat ERK2 kinase (RATERK2). Sequences were aligned by the MULTALIGN program; identities are shaded, positions where three of the four sequences match are noted in the consensus, and residues found in all four proteins are underlined in the consensus. Dashes represent gaps introduced to maximize the alignments. Nonhomologous regions at the N termini have been omitted. This region of CEK1 is rich in alanine and glutamine, including a polyglutamine tract that is polymorphic in two clones: M152p8 contained 13 glutamines starting at aa 34, while M161p11 contained 5. The M161p11 sequence (1681 bp) encodes 417 aa.

DISCUSSION

Complementation of *S. cerevisiae* mutations can allow the identification of homologous genes from a variety of organisms that are less experimentally tractable (2, 3). Here we have shown that the selection of heterologous gene products that dominantly interfere with a cellular pathway can provide an alternative functional assay for genes that are involved in related activities.

We have used this approach to identify six sequences from *C. albicans* that interfere with proper pheromone-mediated cell cycle arrest in *S. cerevisiae* and thus allow pheromone-supersensitive cells, which are defective in the *SST1* and *SST2* functions, to divide in the presence of α-factor. This interference could be mediated through one of several possible mechanisms. Introduction of a gene encoding an α-factor-degrading protease could replace the missing *SST1* function (the BAR1 protease) and reduce pheromone response by degrading α-factor (27), but none of the transformants were found to contain such an α-factor-specific protease activity. The *SST2* function is required for proper adaptation to the presence of pheromone (39), and introduction of a *C. albicans* sequence that could stimulate such adaptation would

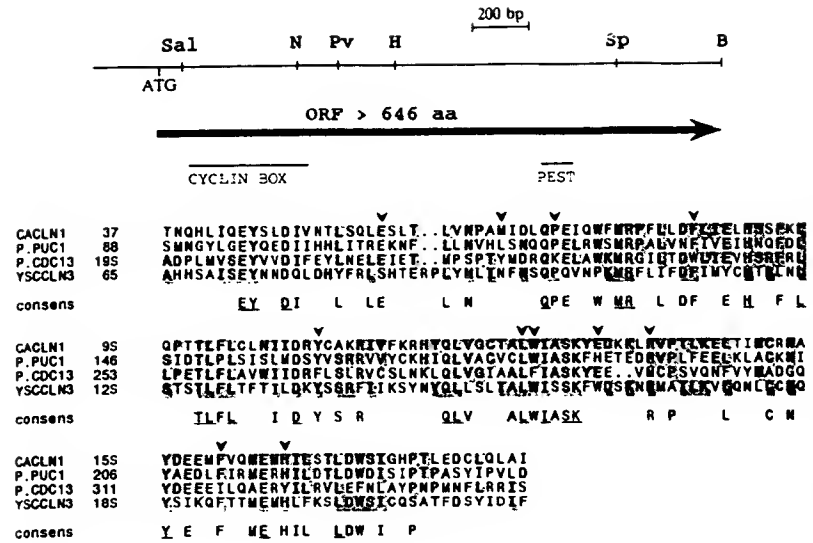

FIG. 3. The *C. albicans CCN1* gene. (*Upper*) Restriction map. B, *Bam*HI; H, *Hin*dIII; N, *Nsi* I; Pv, *Pvu* II; Sal, *Sal* I; Sp, *Spe* I. The cyclin box region and a PEST sequence (31) are noted. This gene maps to *C. albicans* chromosome 5. (*Lower*) Comparison of the cyclin boxes of CCN1 (CACLN1) with *S. cerevisiae* CLN3 (YSCCLN3) (15, 31) and *Sch. pombe* cdc13 (P.CDC13) (32) and puc1 (P.PUC1) (33). Dots represent gaps introduced to maximize the alignments. The residues believed to differentiate G_1 and G_2 cyclins are marked with arrowheads; CCN1 has the G_1-specific residue at most of these sites. The M166p16 sequence (2175 bp) encodes 646 aa.

also reduce pheromone responsiveness. However, none of the plasmids appeared to stimulate adaptation; the transformants were either largely insensitive to pheromone or responded by continuing to divide slowly. There was no evidence for an initial arrest followed by a return to normal proliferation that would characterize an adaptive process.

Alternatively, the *C. albicans* sequences could interfere with pheromone-mediated cell cycle arrest either by blocking the transmission of the pheromone-induced signal or by modifying the ability of the cell cycle machinery to respond to this signal. These two possibilities can be distinguished by assessing whether the plasmids interfere with cell cycle arrest specifically or whether they affect other aspects of the mating response, including induction of pheromone-responsive genes such as *FUS1* (40, 41). None of the plasmids had a dramatic effect on the expression of *FUS1*, and thus they primarily appeared to affect cell cycle arrest.

Plasmid M157p4, encoding a potential transcription factor, CZF1, and plasmid M167p11, encoding a RHO-like small GTP-binding protein, CRL1, caused only moderate resistance to pheromone-mediated arrest. The DNA-binding transcription factor STE12 is essential for proper pheromone response (14), and thus, if CZF1 binds to sequences that are also the target of STE12, the presence of CZF1 could interfere with proper cell cycle arrest. No small GTP-binding protein has been identified as playing a role in the pheromone response, and therefore the mechanism of interference of

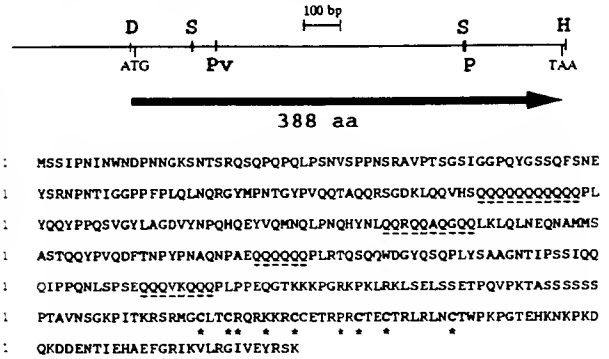

FIG. 4. The *C. albicans CZF1* gene. (*Upper*) Restriction map. D, *Dra* I; H, *Hin*dIII; P, *Pst* I; Pv, *Pvu* II; S, *Sac* I. This gene maps to *C. albicans* chromosome 4. (*Lower*) The four glutamine-rich tracts are underlined: aa 108–118 (11/11), aa 158–167 (7/10); aa 204–209 (6/6); aa 252–259 (6/8). The cysteines and other conserved residues in the putative zinc finger region are shown by stars. The M157p4 sequence (1309 bp) encodes 388 aa.

CRL1 is unclear. Clearly, fortuitous interference may occur, and determination of the biological role of the interfering protein, either by identifying a functional analogue in *S. cerevisiae* or by studying the protein's function directly in *C. albicans*, will be important.

Other plasmids encoded homologues of cell cycle-regulating genes. Plasmids M152p8 and M161p11 encoded an ERK-like kinase, CEK1, and plasmid M166p16 encoded a G_1 cyclin homologue, CCN1. The endogenous *S. cerevisiae* G_1 cyclins and ERK kinases KSS1 and FUS3 function in control of the cell cycle. The *CLN* gene products must be inactivated to allow proper cell cycle arrest in response to pheromones; truncated CLN proteins that have lost sequences required for their degradation and are thus stable act in a dominant

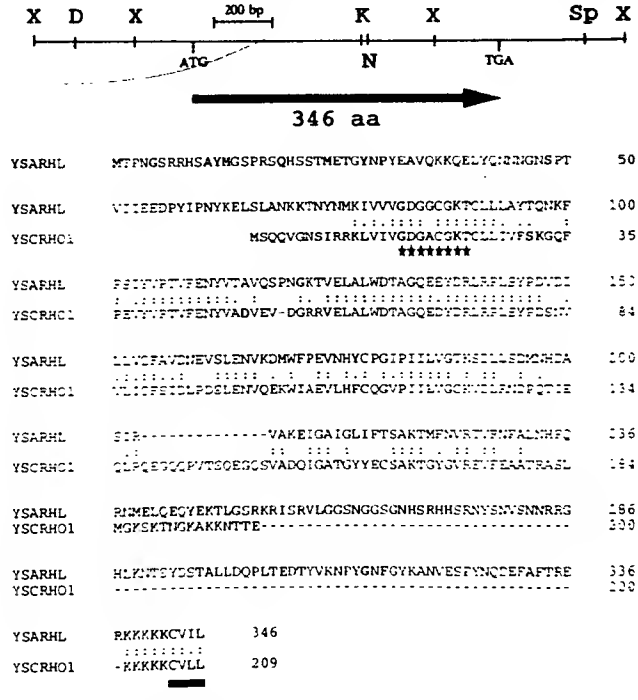

FIG. 5. The *C. albicans CRL1* gene. (*Upper*) Restriction map. D, *Dra* I; K, *Kpn* I; N, *Nsi* I; Sp, *Spe* I; X, *Xba* I. (*Lower*) CRL1 (YSARHL) is compared with the sequence of the *S. cerevisiae* RHO1 protein (YSCRHO1) (38). Colons show sequence identity, and dots show conservative changes. Residues of the highly conserved GTP-binding domain are shown by stars, and the CAAX box residues are underlined. The M167p11 sequence (2011 bp) encodes 346 aa.

manner to generate pheromone-unresponsive cells (15, 31). The *C. albicans* cyclin acts similarly to such mutant *S. cerevisiae* cyclins and creates pheromone-unresponsive cells that initiate cell division at a smaller than normal size. This occurs either because the C-terminally truncated *CCN1* gene product is stable or because the *S. cerevisiae* inactivation signals are not recognized by the *C. albicans* cyclin. Thus *CCN1* disrupts normal pheromone-mediated cell cycle arrest by providing a G_1 cyclin function that cannot be inactivated by the pheromone response pathway.

The functions of the *FUS3* and *KSS1* gene products are less well defined than those of the CLN cyclins. However, the phenotype of pheromone resistance resulting from either *FUS3* inactivation or *KSS1* overexpression is reported to depend on *CLN3* (17, 28), implying a connection between these kinases and the cell cycle regulatory machinery. Overproduction of either CEK1 or KSS1 produces a similar pheromone-insensitive phenotype, and the two proteins are structurally similar. CEK1, however, cannot substitute for KSS1 (or for FUS3); CEK1 transformants of a *kss1 fus3* strain remained sterile. This property would have precluded the identification of *CEK1* by classical complementation strategies and highlights the utility of the identification of dominant negative natural variant genes as a valuable adjunct to direct complementation approaches.

The *C. albicans* dominant negative natural variant genes were readily identified from a genomic library; the 6 independent interfering sequences were identified from an initial screen of ≈2000 transformants. Based on estimates of the *C. albicans* genome being similar in size to the genome of diploid *S. cerevisiae* (18), and the average size of the plasmid inserts being 8 kilobases, these transformants should represent a significant fraction of the entire genome. Indeed, we found overlapping clones for two of the genes that we isolated. However, subsequent screens have identified new interfering genes, so the initial screen did not identify all the possible dominant negative natural variant genes. Similar screens using *S. cerevisiae* sequences have identified three high-copy suppressors of pheromone-supersensitive strains, *SST2* (39), *SCG1* (16), and *KSS1* (17). Thus it appears that selection of heterologous genes that create pheromone resistance through interfering with some aspect of pheromone-mediated cell cycle arrest can be efficient relative to similar selections involving homologous genes.

The selection of functionally related proteins through dominant negative selection can be extended. Mammalian cDNAs have been identified that can interfere with RAS-mediated signaling in *S. cerevisiae* (42). Thus genes from different eukaryotes can be selected through interference with *S. cerevisiae* cellular functions. It should also be possible to extend this approach to selecting interfering functions in other transformable systems: for example, genes from lower eukaryotes could provide interfering functions in mammalian cells, or genes normally expressed in one mammalian tissue may interfere when expressed in a different cell type. Because dominant negative mutations exert their influence even in the presence of functional protein, it is not necessary to have a mutation in the target cellular pathway to identify proteins that function in that pathway. Thus, selection of dominant negative functions from heterologously expressed libraries should provide a simple and powerful strategy to investigate complex cellular processes and to identify by function genes from genetically intractable systems.

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